CLINICAL AND LABORATORY ASPECTS OF INTIMAL HYPERPLASIA IN LOWER LIMB BYPASS GRAFTS

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Paul Dunlop

August 1995

For Monica and James

Science proceeds by successive answers to questions more and more subtle, coming nearer and nearer to the very essence of phenomena.

(Louis Pasteur 1822-1895)

ABSTRACT

This thesis examines aspects of intimal hyperplasia in infrainguinal bypass grafts. There are 3 introductory chapters. Chapter 1 describes the aetiology and treatment of lower limb vascular disease, with particular reference to atherosclerosis. Chapter 2 is a review of the literature regarding the vascular biology of intimal hyperplasia. Chapter 3 is a review of the techniques of cell and organ culture used in the study of vascular disorders.

Chapter 4 is composed of four clinical studies of infrainguinal grafts performed at the Leicester Royal Infirmary. There is a prospective study of the long-term benefit of vein graft surveillance. This is followed by a study of the long-term results of percutaneous transluminal angioplasty of vein graft stenoses. A retrospective review of the outcome of polytetrafluoroethylene (PTFE) grafts used in lower limb bypass surgery forms the 3rd part of the chapter. This is followed by a prospective study of the benefits of graft surveillance for synthetic grafts.

The next 3 chapters are laboratory based. Chapter 5 looks at the effect of various growth factors on the proliferation of human saphenous vein smooth muscle cells, the cells involved in the intimal hyperplasia of vein graft stenoses. Chapter 6 looks at aspects of platelet-derived growth factor on the proliferation seen in an organ culture model of human saphenous vein. Chapter 7 examines 2 different ways of incorporating flow in an organ culture of human saphenous vein and compares the effects of high and low shear rates on the development of intimal hyperplasia in the model.

In the final chapter, the experimental results are summarised and possible future work relating to the thesis is discussed.

Publications and presentations arising from this thesis to date

Papers

1. Percutaneous transluminal angioplasty of infrainguinal vein graft stenosis: long-term outcome
   P. Dunlop, K. Varty, T. Hartshorne, P.R.F. Bell, A. Bolia, N.J.M. London
   British Journal of Surgery 1995; 82: 204-206

2. The fate of infrainguinal PTFE grafts and an analysis of factors affecting outcome.
   R.D. Sayers, M.M. Thompson, P. Dunlop, N.J.M. London, P.R.F. Bell
   European Journal of Vascular Surgery 1994; 8: 607-610

3. The long-term outcome of infrainguinal vein graft surveillance
   P. Dunlop, T. Hartshorne, A. Bolia, P.R.F. Bell, N.J.M. London
   European Journal of Vascular Surgery - accepted - in press.

Submitted papers

1. The effect of a surveillance programme on the patency of synthetic infrainguinal bypass grafts
   P. Dunlop, H. Handford, T. Hartshorne, A. Thrush, P.R.F. Bell, N.J.M. London.
   European Journal of Vascular Surgery

Published abstracts

1. The effect of recombinant cytokines on human saphenous vein smooth muscle cell growth.
   P. Dunlop, K. Varty, K.E. Porter, P.R.F. Bell, N.J.M. London.
   Surgical Research Society, Glasgow, 7th July 1994.
   British Journal of Surgery 1994; 81: 1806

2. The long-term outcome of angioplasty of vein graft stenoses.
   P. Dunlop, K. Varty, T. Hartshorne, P.R.F. Bell, A. Bolia, N.J.M. London.
   International Angiology 1995; 14 (Suppl I): 259

Oral presentations

1. The fate of PTFE grafts and the need for graft surveillance.
   East Midlands Surgical Society, Sheffield, 29 Oct 1993.

2. Is angioplasty of infrainguinal vein graft stenoses a durable procedure?
   Association of Surgeons of Great Britain and Ireland, 20th-22nd April 1994.

3. The effect of recombinant cytokines on human saphenous vein smooth muscle cell growth.
Surgical Research Society (Patey Prize session), Glasgow, 7th-8th July 1994.

4. Is angioplasty of infrainguinal vein graft stenoses a durable procedure?
Vascular Surgical Society of Great Britain and Ireland, Edinburgh, 24th-25th November 1994.

5. The long-term outcome of angioplasty of vein graft stenoses.
International Union of Angiology, London, 3rd-7th April 1995.

6. Is surveillance of synthetic infrainguinal grafts worthwhile?
Midlands Vascular Surgical Society, Leicester, 17th March 1995.

7. Vein graft surveillance needs to continue beyond 12 months.
Midlands Vascular Surgical Society, Leicester, 17th March 1995.

Poster presentations

1. The effect of recombinant cytokines on human saphenous vein smooth muscle cell growth.
P. Dunlop, K. Varty, K.E. Porter, P.R.F. Bell, N.J.M. London.
VIII International Conference on Vascular Cell Biology, Heidelberg, September 1st 1994.

2. Vein graft surveillance needs to continue beyond 12 months.
P. Dunlop, T. Hartshorne, A. Bolia, P.R.F. Bell, N.J.M. London.
Association of Surgeons of Great Britain & Ireland, London, 31st June-2nd May 1995.

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Finally, Professor P.R.F. Bell for allowing me the opportunity to carry out research in his Department and for his constant support and encouragement.

List of abbreviations

ABPI	Ankle Brachial Pressure Index
ACE	Angiotensin Converting Enzyme
ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BAEC	Bovine Aortic Endothelial Cell
bFGF	Basic Fibroblast Growth Factor
Brd-U	5-Bromo-2-deoxyuridine
BSA	Bovine Serum Albumin
CABG	Coronary Artery Bypass Graft
cAMP	cyclic Adenosine Monophosphate
cGMP	cyclic Guanosine Monophosphate
DNA	Deoxyribonucleic Acid
EC	Endothelial Cell
ECD	European Consensus Document
EDRF	Endothelial Derived Relaxing Factor
EDTA	Diaminoethanetetra-acetic acid
ET-1	Endothelin-1
FCS	Foetal Calf Serum
H+E	Haemotoxylin and Eosin
HB-EGF	Heparin Binding Epidermal Growth Factor
HUV	Human Umbilical Vein
HUVEC	Human Umbilical Vein Endothelial Cell
ICAM	Intercellular Adhesion Molecule
IFN-γ	Interferon gamma
IGF-1	Insulin-like Growth Factor-1
IL-1	Interleukin-1
IMA	Internal Mammary Artery

kD	kilodalton
LDL	Low Density Lipoprotein
MEM	Minimal Essential Medium
MMP	Matrix Metalloproteinase
mRNA	messenger Ribonucleic Acid
PDGF	Platelet-Derived Growth Factor
PTA	Percutaneous Transluminal Angioplasty
PTFE	Polytetrafluoroethylene
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RPMI	Roswell Park Memorial Institute
SMA	Smooth Muscle Actin and Miller's Elastin
SMC	Smooth Muscle Cell
T/E	Trypsin and EDTA
TGF-β	Transforming Growth Factor beta
TNF-α	Tumour Necrosis Factor alpha
VCAM	Vascular Cell Adhesion Molecule
VLDL	Very Low Density Lipoprotein

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Introductory overview

Stenoses occur in up to 30% of infrainguinal vein bypass grafts and represent the commonest cause of graft failure between 1 month and 1 year after surgery. This thesis examines various aspects of these stenoses and the processes involved in their aetiology. The first 3 chapters are reviews of the literature, the fourth chapter contains several clinical studies and Chapters 5-7 are laboratory studies.

Chapter 1 reviews the causes and management of lower limb vascular disease. There is a section devoted to the aetiology and pathogenesis of atherosclerosis and a summary of other less common causes of lower limb vascular disease. The presentation of both acute and chronic lower limb ischaemia is discussed. The various treatment options including conservative management, endovascular techniques and operative surgery are examined. Included within the description of surgical treatment options for chronic ischaemia is an explanation of vein graft surveillance to detect vein graft stenoses. Chapter 1 concludes with a section outlining current concepts regarding the aetiology of vein graft stenoses.

Chapter 2 considers the vascular biology of intimal hyperplasia. The cells involved in the process, the growth factors known to exert an effect and the influence of flow on intimal hyperplasia are discussed. There is also a summary of experimental work suggesting possible pharmacological or molecular biological manipulation of intimal hyperplasia.

Chapter 3 summarises the laboratory techniques used to isolate and culture vascular smooth muscle cells (SMCs) and describes the organ culture method for studying vascular tissue.

Chapter 4 consists of clinical studies of venous and prosthetic grafts performed at the Leicester Royal Infirmary. Whilst vein graft surveillance is widely accepted as being of benefit in the first year after surgery, there have been few *long-term* studies of the effect of a surveillance programme. Therefore the first part of the chapter looks at the long-term effects of such a programme on the overall patency of vein grafts performed at the Leicester Royal

Infirmary. Analysis of the programme reveals a marked difference between primary and primary assisted patency suggesting that graft surveillance does significantly improve the long-term patency of vein grafts by detecting stenoses at an early stage.

Percutaneous transluminal angioplasty (PTA) is the accepted first line treatment of vein graft stenoses in most centres including the Leicester Royal Infirmary. Whilst PTA is an effective treatment for graft stenoses, there are few reports of the long-term patency of these stenoses following PTA. The second part of chapter 4 is a prospective study of 33 stenoses which have undergone PTA and are then followed up at regular intervals in a surveillance clinic. The median follow up time is 39 months.

The third part of Chapter 4 is a retrospective review of all the PTFE grafts performed at the Leicester Royal Infirmary over a 5 year period from January 1988 to March 1993. The study revealed no significant difference between the primary and primary assisted patencies of the PTFE grafts. This contrasts with vein grafts where the dedicated surveillance programme produces an improved primary assisted patency. There have been very few studies of the effectiveness of surveillance for synthetic grafts, so the fourth part of Chapter 4 is an analysis of a synthetic graft surveillance programme.

Although there are numerous studies on the effect of various growth factors on the proliferation of animal smooth muscle cells (SMCs), there are few studies looking at human tissue. Chapter 5 examines the effects of various recombinant growth factors on the proliferation of human saphenous vein SMCs, the predominant cell type in vein graft stenoses. Previous work in our Department has revealed a paracrine mediator which is able to promote intimal hyperplasia in a denuded segment of saphenous vein. The experiments in Chapter 5 show that Platelet-Derived Growth Factor (PDGF) is by far the most stimulatory growth factor tested in promoting growth in the human saphenous vein SMCs.

In Chapter 6, the significance of PDGF in the organ culture model of intimal hyperplasia is further studied. Recombinant PDGF is added to denuded segments of vein to assess its ability to induce neointima formation in the presence of medium containing 30%

foetal calf serum (FCS). This addition of exogenous PDGF has no effect on intimal SMC proliferation. However, in an additional experiment to look at the importance of PDGF in the model, a blocking antibody to PDGF is used. This antibody is placed in the medium of a coculture model containing both an intact segment of vein and a denuded segment of vein. In the control experiments, neointimal proliferation is induced in the denuded segment of vein by the presence of the intact vein. This effect is abolished in the presence of the anti-PDGF antibody, implying that PDGF does have an effect in the model. In addition to the above 2 experiments, the proliferative effect of the conditioned medium from the organ culture model was studied using a bioassay system. The ability of the medium to stimulate incorporation of tritiated thymidine into 3T3 fibroblast cells was assessed.

Chapter 7 investigates the effects of flow on intimal hyperplasia in the organ culture model. In the first part of the Chapter there is a description of a flow model incorporating an intact segment of vein in series within a flow system. This particular model had several technical problems and was not suitable for comparing paired segments of vein. Therefore a flow model was used which incorporated segments of vein pinned out within a piece of silicone tubing. This second flow system was more artificial than the first but it did allow comparison of paired segments of vein at different shear stresses. Chapter 8 summarises the contents of the thesis and discusses future work related to the findings of the thesis.

CHAPTER 1 - Lower limb vascular disease and treatment

1 a) ATHEROSCLEROSIS

i) INTRODUCTION

ii) RISK FACTORS

iii) PATHOGENESIS

1 b) LOWER LIMB VASCULAR DISEASE

i) INTRODUCTION

ii) INTERMITTENT CLAUDICATION

iii) CRITICAL ISCHAEMIA

iv) ACUTE ISCHAEMIA

v) OTHER CAUSES OF LOWER LIMB VASCULAR DISEASE:

Thromboangiitis obliterans

Popliteal entrapment

Cystic medial necrosis

Radiation

Thrombocythaemia

1 c) TREATMENT OF LOWER LIMB VASCULAR DISEASE

i) TREATMENT OF ACUTE ISCHAEMIA:

Fibrinolysis

Surgical

ii) TREATMENT OF CHRONIC ISCHAEMIA;

Conservative

Endovascular:

Angioplasty

Stents

Atherectomy

Surgical:

Endarterectomy

Arterial bypass surgery

Vein grafts; Surveillance, Treatment of vein graft stenoses

Synthetic grafts

1 d) CURRENT CONCEPTS OF VEIN GRAFT STENOSIS

1 a) ATHEROSCLEROSIS

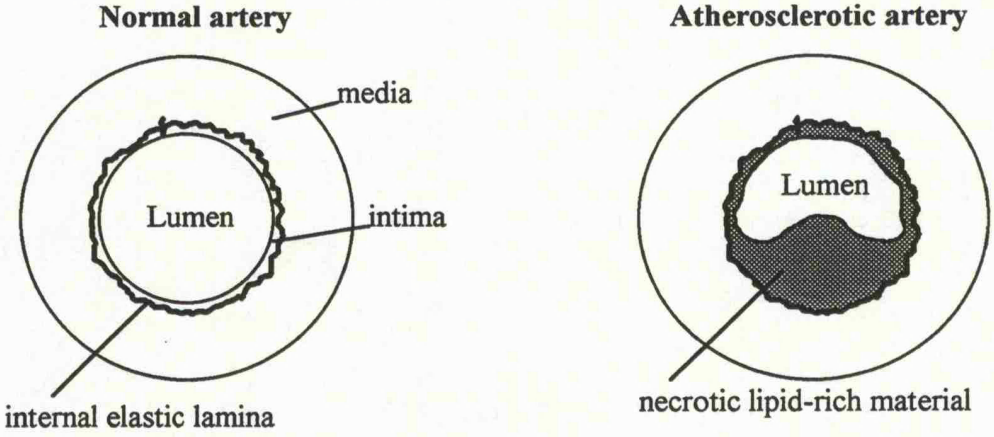
1 a.i) INTRODUCTION

Atherosclerosis is a disease process affecting arterial blood vessels. There is narrowing of the arterial lumen, which may be accompanied by thrombosis. There is patchy deposition of lipid material and fibrosis within the intima of the vessel wall. This combination of soft, lipid-rich material (*athere* = porridge) and fibrosis (*sclerosis* = hardness) gives the process its Greek-derived name. Atherosclerosis is the commonest cause of mortality in the developed world. Ischaemic heart disease alone accounted for 145,904 deaths in 1992 in England and Wales (Office of Population Censuses and Surveys 1993). This represents about 30% of all male deaths and 22% of all female deaths. Atherosclerosis in the carotid, vertebral and basilar arteries is responsible for much cerebrovascular disease as it predisposes to thrombosis and to embolic disease. Atherosclerotic disease in the aorta may predispose to the formation of an aortic aneurysm. Both the incidence of and the mortality from aortic aneurysm have risen this century despite improved diagnosis and treatment (Johansson *et al.* 1994, Nasim *et al.* 1995). Atherosclerosis in the lower limb is usually referred to as peripheral vascular disease. This may manifest as intermittent claudication, with pain in the calf or buttock on walking which is relieved by rest. In more advanced disease, there may be constant pain in the limb or gangrene may be present. In such instances, the blood flow to the limb needs to be improved or amputation may be necessary.

The incidence of atherosclerotic disease rises with increasing age. It is likely that the lifestyle adopted in early childhood affects the later development of atherosclerosis (Haust 1990). Lipid deposits can be seen in the aorta of children and young adults in the form of *fatty streaks*. These are irregular yellow areas on the luminal surface of the vessel. Histologically, they consist of accumulations of lipid droplets in smooth muscle cells and macrophages within the intima of the blood vessel. Fatty streaks are an almost universal finding and occur even in communities where there is a very low incidence of atherosclerotic disease. The significance of these lesions is therefore unknown. In those communities where atherosclerosis is common, fibro-lipid plaques start to appear in early adulthood. They are more elevated than fatty streaks

and the intima is significantly thickened. There is a proliferation of the smooth muscle cells with an accumulation of both intracellular and extracellular lipid. As these lesions progress, there is necrosis and liquefaction within the plaque often with subsequent calcification. There may be thinning of the media of the artery which will predispose to aneurysm formation.

Figure 1a.1 Diagram of atherosclerotic blood vessel. There is considerable intimal thickening in the atherosclerotic vessel with narrowing of the lumen.



1 a.ii) RISK FACTORS

Atherosclerosis is more prevalent in developed countries, particularly among certain socio-economic groups, and is particularly prominent in certain families. It has an association with metabolic disorders such as diabetes mellitus and familial hyperlipidaemias. There is some debate about the effect of dietary intake of lipids and the value of exercise in preventing atherosclerosis. There is less debate regarding the effect of tobacco smoking and its ability to promote atherosclerosis.

In England and Wales, atherosclerosis is especially prevalent in lower socio-economic groups. The mortality from ischaemic heart disease has not fallen appreciably in these groups despite a generalised decrease in the incidence of atherosclerotic disease in the population. The reasons for this are multifactorial. Smoking has declined in the past 20 years but this decline has been less in manual than in non-manual workers (HMSO 1994). Dietary factors may have a role (Denke 1994, Mera 1993) as overall saturated fat intake is less in social classes I and II.

Also, regular vigorous exercise, which may have a cardioprotective effect, is more common in higher social classes.

Genetic factors are almost certainly involved. The familial hyperlipidaemias are examples of inherited disorders which predispose to early accelerated atherosclerosis. However, even where there hyperlipidaemia is not present, there are often higher than expected incidences of atherosclerotic disorders within family groups. There is almost certainly a polygenic influence in addition to environmental factors which affect the whole family group.

Male gender is a risk factor. Men are much more likely to die from coronary artery disease than women and are much more likely to suffer from other atherosclerotic diseases. This gender difference decreases with advancing age after the menopause suggesting that female hormones may have a protective effect.

Familial hyperlipidaemias types IIa, IIb, III, and IV are associated with accelerated atherosclerosis. Vascular disease occurs at a very early age in patients with these disorders. Elevated serum levels of cholesterol, low density lipoprotein (LDL) and very low density lipoprotein (VLDL) have been shown to be associated with atherosclerosis in epidemiological studies. A reduction of the serum cholesterol, LDL and VLDL in hyperlipidaemic patients is able to improve the mortality from ischaemic heart disease. However, the reduction of serum levels of these lipid fractions in normal individuals has not been shown to prevent atherosclerosis. Those familial hyperlipidaemias known to be associated with accelerated atherosclerosis in humans are shown in Table 1a.1.

Table 1a.1 Familial hyperlipidaemias with an elevated risk of atherosclerosis

	Frederickson Classification	Inheritance (where known)
Familial hypercholesterolaemia	IIa, IIb	Autosomal dominant
Familial Combined hyperlipidaemia	IIa, IIb, IV	
Remnant hyperlipoproteinaemia	III	
Common hyperlipidaemia	IIa	Polygenic

Diabetes mellitus is a disorder of insulin production leading to elevated serum levels of glucose. In type I diabetes mellitus, there is a complete absence of insulin production and in type II diabetes mellitus, there is a relative reduction of insulin production or there is relative insulin-resistance. Both types of diabetes mellitus are associated with accelerated atherosclerosis (Schwartz *et al.* 1992). Arterial disease occurs at an earlier age in diabetics. The large vessel disease of atherosclerosis is compounded by small vessel disease and peripheral neuropathy. A diabetic patient with peripheral vascular disease is at greater risk of developing gangrene and of losing a limb than a non-diabetic patient. The results of arterial surgery are worse in diabetics in some series (Prendiville *et al.* 1990) although in Leicester, Sayers et al. showed that diabetes was not an indicator of poor surgical outcome but there was increased perioperative mortality (Sayers *et al.* 1993b).

Tobacco consumption is strongly associated with atherosclerosis (McGill 1990, Diana 1990). Indeed, it is considered to be the single most important preventable factor in atherosclerosis (HMSO 1994). Cigarette smoking is thought to promote atherosclerosis by causing direct damage to vascular endothelium and by increasing platelet aggregability. The production of von Willebrand factor, a potent aggregator of platelets, is increased in vascular endothelial cells in response to smoking (Blann *et al.* 1994). Nicotine itself causes decreased production of prostacyclin by cultured rabbit aortic smooth muscle cells and increases thromboxane B2 in rats (Hui *et al.* 1992) (Prostacyclin is a potent vasodilator which inhibits platelet aggregation). Decreased production of prostacyclin has also been shown in umbilical arteries derived from smoking mothers as compared to non-smoking mothers (Ahlsten *et al.* 1990).

One of the first treatment options in peripheral vascular disease (and ischaemic heart disease) is to persuade the smoking patient to give up his habit. In many individuals, there will be a significant symptomatic improvement. However, some patients are unable or unwilling to do this. Some surgeons have argued that surgery should not be routinely offered to such patients as they have already failed to comply with the first treatment option (Underwood *et al.* 1993). Also, arterial bypass surgery has a higher failure rate in most series where the patient

continues to smoke after his surgery (Provan *et al.* 1987, Ameli *et al.* 1988, Wiseman *et al.* 1989, Ameli *et al.* 1989, Stonebridge *et al.* 1994, Powell *et al.* 1992, Wiseman *et al.* 1990, Prendiville *et al.* 1990). This is principally because of the increased thrombotic tendency in smokers. There is also evidence that smokers' veins already have some pre-existing disease (Higman *et al.* 1994) and smoking impairs endothelial-dependent relaxation of saphenous vein (Higman *et al.* 1993).

1a.iii) PATHOGENESIS

The main constituents of the atherosclerotic plaque are smooth muscle cells and lipids. Both the deposition of lipids and the proliferation of vascular smooth muscle cells have a part in the progression of atherosclerosis. In the past, several theories have been put forward to explain atherosclerosis. A 'response to injury' hypothesis was first put forward by Virchow (Virchow 1856), and a monoclonal smooth muscle cell hypothesis (Benditt *et al.* 1973) became prominent in the 1970's. More recently, it has become accepted that injury can lead to both smooth muscle cell proliferation and infiltration of inflammatory cells and lipid-rich material via the action of various growth factors (Ross 1993).

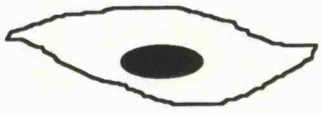
In a comprehensive review of atherosclerosis by Ross (Ross 1993), the growth factors thought likely to be important in promoting atherosclerosis in man were platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), heparin-binding epidermal growth factor (HB-EGF), insulin-like growth factor-1 (IGF-1), interleukin-1 (IL-1), Tumour Necrosis Factor-α (TNF-α) and transforming growth factor-β (TGF-β). These growth factors can all be released as part of the 'response to injury' and their expression is upregulated in atherosclerotic as opposed to normal arteries. The growth factors and the cells responsible for producing them are shown in Figure 1a.2.

The initiator of atherosclerosis is probably an injury to the endothelium. The endothelium does not need to be destroyed but merely needs to be 'dysfunctional' to allow the passage of lipoprotein (Mora *et al.* 1987) and the migration of macrophages and T-cell lymphocytes into the subintimal space. 'Normal' endothelium is far from being a passive lining for blood vessels. In addition to providing a non-thrombogenic, permeable exchange barrier,

the endothelium produces vasodilatory chemicals such as Endothelium-Derived Relaxing Factor (EDRF) and prostacyclin (PGI_2) and the potent vasoconstrictor endothelin. It produces various growth factors and cytokines such as PDGF, bFGF, IL-1, TNF-α, Macrophage Colony Stimulating Factor (MCSF) and Monocyte Chemotactic Protein-1 (MCP-1). Altered production of these growth factors and cytokines can occur in 'dysfunctional' endothelium predisposing to atheroma formation. The endothelium is also able to oxidise lipoproteins. Oxidation of LDL produces oxLDL, a free radical which is toxic for endothelial cells, smooth muscle cells and fibroblasts (Munro *et al.* 1988). Oxidised LDL is taken up by macrophages. These fat-laden cells are the characteristic foam cells seen in atherosclerotic lesions (Yla-Herttuala *et al.* 1989, Steinberg *et al.* 1989). Endothelial Derived Relaxing Factor is able to neutralise ox-LDL but EDRF production may be diminished in dysfunctional endothelium.

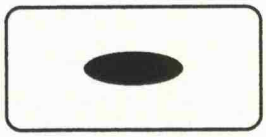
Vascular smooth muscle cell proliferation is an integral feature of atherosclerosis. Benditt's monoclonal theory (Benditt *et al.* 1973) even suggested that each atherosclerotic lesion was derived from a single progenitor smooth muscle cell. SMCs are the predominant cell type in atherosclerotic lesions and are mainly of the synthetic phenotype (Ross 1993). They are therefore more responsive to growth factors and cytokines, they can produce their own growth factors and can secrete extracellular matrix into the atherosclerotic lesion. Arterial SMCs are particularly sensitive to bFGF and PDGF. Platelets adherent to the atherosclerotic plaque will release PDGF-AB, and endothelial cells can produce PDGF-BB. Both isomers of PDGF will stimulate the SMCs to proliferate and also to produce PDGF-AA (Libby *et al.* 1988b). This autocrine production of PDGF-AA by the SMCs can further stimulate proliferation. SMCs contain bFGF but do not secrete it. However, if SMC injury or death occurs, bFGF can be released and could further stimulate SMC proliferation. Other growth factors present in atheromatous plaques or produced by platelets which can stimulate SMC proliferation include IL-1, IGF-1, TNF-α, TGF-β, HB-EGF, endothelin, serotonin and thrombospondin.

Figure 1a.2 Cells involved in atherosclerosis and the growth factors and inhibitors produced by those cells (Taken from Ross 1993).



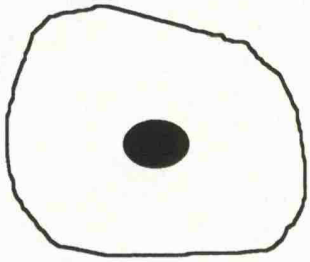
Smooth muscle cell

PDGF-AA
bFGF
TGF β
TNF α
IL-1
IGF-1



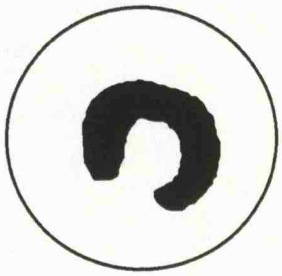
Endothelial cell

PDGF
bFGF
TGF β
IL-1
IGF-1
EDRF



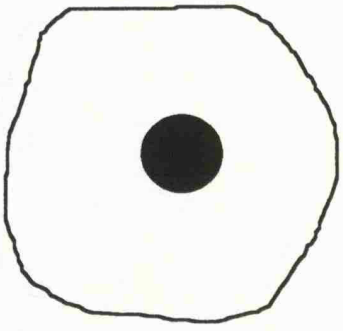
Platelet

PDGF
TGF α
TGF β
IGF-1



Macrophage

PDGF
bFGF
TGF α
TGF β
TNF α
IL-1



T-Lymphocyte

TGF β
TNF α
IL-1
IFN γ

Macrophages and T lymphocytes are present in large numbers in human atherosclerotic plaques (Ross 1986). The presence of the lymphocytes suggests a possible immunological as well as an inflammatory role. However, whilst autoantibodies to oxLDL have been demonstrated, no specific antigen has been shown to be responsible for an immune response in atherosclerosis (Ross 1993). Macrophages are able to degrade the extracellular matrix surrounding vascular smooth muscle cells and promote a change from a contractile to a secretory phenotype capable of proliferation (Campbell *et al.* 1991). This may be one of the initial steps in the development of atherosclerosis in humans.

Interruption of the vasa vasorum in an animal model has been shown to produce lesions resembling atherosclerosis (Martin *et al.* 1990) suggesting that perivascular hypoxia may have a role to play in atherosclerosis. Thus damage to the small vessels could lead to atherosclerosis in the larger vessels. This is one mechanism which may account for the high incidence of atherosclerosis in diabetics who have small vessel disease.

1 b) LOWER LIMB VASCULAR DISEASE

1 b.i) INTRODUCTION

Atherosclerosis can occur in all arteries down to a diameter of about 2mm but it has a predilection for the aorta, particularly its abdominal part, the carotid, vertebral and basilar arteries, the coronary arteries and the lower limb. The most commonly affected segments of the lower limb are: the femoral artery, especially that part in the adductor canal immediately above the knee; the origin of the profunda femoris artery; the popliteal trifurcation and the individual peroneal and tibial arteries. Figure 1b.1 shows an occluded atherosclerotic blood vessel. Peripheral vascular disease is usually benign and only a small percentage of patients will ever come to an amputation (Bell 1988, Silbert *et al.* 1958, Singer *et al.* 1960, Bloor 1961). However, the disease becomes increasingly common with advanced age so that below the age of 60 years it affects about 3% of the population but over the age of 75 years it affects up to 20 % (Vogt *et al.* 1992). Thus, as the proportion of elderly people increases, more people will require treatment.

Figure 1b.1 Transverse section (x40) of atherosclerotic coronary artery occluded with thrombus (Haemotoxylin + Eosin).

1 b.ii) INTERMITTENT CLAUDICATION

Lower limb atherosclerosis usually presents with the symptom of intermittent claudication. Classically, the patient complains of a cramping pain in the calf which comes on after walking a certain, reproducible distance. The pain is relieved by rest, after which he can continue to walk until the pain returns again. The pain is usually in the calf when the femoropopliteal segment is affected but may be in the buttock or thigh if atherosclerosis is present in the aortoiliac segment. Impotence may also be a feature of aortoiliac occlusive disease because of reduced internal iliac artery blood flow. The 'claudicating distance' and the time taken to recover after resting act as a good clinical indicator of the severity of the disease. The diagnosis can usually be made from the history alone although physical examination of the pulses combined with non-invasive measurements such as ankle pressure and Ankle Brachial Pressure Indices (ABPI) can help to distinguish the pain from that caused by arthritic or neurological diseases. Arteriography is regarded as the gold standard of investigation of peripheral vascular disease to delineate the extent of the disease. However, it is invasive and

has a significant morbidity and mortality. Duplex ultrasonography in experienced hands is able to delineate the extent of the disease (Kohler *et al.* 1990) and also, provide information about flow (Zierler *et al.* 1992) and about the haemodynamic significance of stenotic lesions.

1 b.iii) CRITICAL ISCHAEMIA

In most cases, symptoms do not progress beyond those of intermittent claudication and with conservative treatment the symptoms may improve as collateral blood vessels open up to improve blood flow to the lower limb. In a review of the literature by Fowkes, improvement in symptoms occurred in up to half of all patients presenting with intermittent claudication whilst progression to amputation occurred in less than 10% of cases (Fowkes 1988). In those cases where there is progression of disease, the claudicating distance may decrease so much that it severely hinders the patient's normal daily activities. The blood supply to the lower limb may even diminish to such an extent that the tissue is hypoxic at rest. At this point, the patient may have continuous pain which is referred to as 'rest pain'. This is a serious condition as it implies that the limb may become non-viable as its blood supply is not adequate to meet the tissue's needs even at a low metabolic rate. Areas of tissue necrosis may occur at the extremities. Gangrene may then occur due to infection in the necrotic tissue. Prior to the advent of modern surgery and antibiotics this meant amputation and probable death. Even today, there are some 10,000 amputations per annum in the United Kingdom for vascular insufficiency (Cheshire *et al.* 1992). The presence of rest pain, tissue necrosis or gangrene is referred to as 'critical ischaemia' because of the risk of limb loss without intervention. The European Working Group on Critical Leg Ischaemia defined chronic critical leg ischaemia more precisely as "*Persistently recurring ischaemic rest pain requiring regular adequate analgesia for more than 2 weeks, with an ankle systolic pressure ≤50mmHg and/or a toe systolic pressure of ≤30mmHg; or ulceration or gangrene of the foot or toes, with an ankle systolic pressure ≤50mmHg and/or a toe systolic pressure of ≤30mmHg*" (European Working Group on Critical Leg Ischaemia 1991). This attempt to standardise the definition was intended to make the comparison of clinical trials from different centres more meaningful. However, the clinical usefulness of the ECD definition is in doubt as it has been shown that patients with clinical critical ischaemia who do not fulfil the ECD criteria fare as badly as those who do (Thompson *et al.* 1993).

1 b.iv) ACUTE ISCHAEMIA

Occasionally, there may be a rapid deterioration of the limb usually due to thrombosis in a previously diseased atherosclerotic segment of artery. Embolism associated with atrial fibrillation, mural thrombus after a myocardial infarct, rheumatic valve vegetations, an atrial myxoma or an aortic aneurysm can also lead to an acutely ischaemic limb. The acute event occurs too quickly for collateral vessels to open up and the patient presents with a painful, pallid, paraesthetic, pulseless, poikilothermic limb ('The 5 Ps'). If the blood flow is not restored within 6 hours of the event, there is a high risk of limb loss.

Rheumatic heart disease is now relatively rare and atrial fibrillation is often well controlled medically so that these causes of embolism occur less frequently. In addition, with the general increase in longevity, the prevalence of atherosclerosis within the population has increased significantly over the past 30 or 40 years. Thrombosis occurring in previously diseased vessels is therefore becoming a more common cause of acute ischaemia.

1 b.v) OTHER CAUSES OF LOWER LIMB VASCULAR DISEASE

Atherosclerosis is the commonest cause of lower limb ischaemia but other disease processes need to be considered particularly in younger individuals.

Thromboangiitis obliterans

Thromboangiitis obliterans (or Buerger's disease) typically affects young male heavy smokers. The disease affects medium size arteries in both upper and lower limbs usually distal to the brachial and popliteal arteries. Histologically, there is an acute inflammatory process within the arterial wall with neutrophil infiltration. There is thrombosis, organisation and recanalisation of the affected vessels. There is often fibrosis of the surrounding connective tissue which may incorporate neighbouring nerves and veins. Distal ulceration or gangrene is common and reconstructive surgery is rarely of any benefit. Continued heavy smoking invariably leads to amputation.

Popliteal entrapment

Popliteal entrapment syndrome occurs where there is repeated compression of the popliteal artery during exercise because of an anomalous relationship of the artery with the tendon of the medial head of the gastrocnemius muscle. There may be an abnormal insertion of the tendon or the artery may follow an aberrant course. It is especially prevalent among young healthy males. Treatment is surgical. Division of the medial head of the gastrocnemius muscle is curative in the early stages. If the entrapment has been long-standing and there is aneurysmal dilatation of the artery, then it may be necessary to bypass the affected segment of artery using a vein graft.

Cystic medial necrosis

This process is characterised by the development of multiloculated cysts in the media of the popliteal artery. It is traumatic in origin and mainly affects young men.

Radiation

This is an uncommon cause of vascular disease. Exposure to excessive doses of radiation can lead to atrophy of blood vessels with subsequent vascular insufficiency.

Thrombocythaemia

Thrombocythaemia impairs blood flow to the lower limbs in two ways. Increased plasma viscosity leads to sluggish microcirculatory flow which impairs tissue oxygenation. Thrombosis in situ is also more likely to occur leading to a further reduction in blood flow.

1 c) TREATMENT OF LOWER LIMB VASCULAR DISEASE

1 c.i) TREATMENT OF ACUTE ISCHAEMIA.

As mentioned above, the 2 principal causes of acute ischaemia are thrombosis and embolism. It is important to determine the cause of the ischaemia as the treatment of the 2 conditions is different. The first line treatment for thrombosis is dissolution of the clot followed

by rectification of the causes leading to thrombosis. An embolism needs to be removed by surgical methods before treatment of the cause of the embolism.

Fibrinolysis

Dissolution of thrombus is referred to as fibrinolysis or thrombolysis. There are 2 principal agents used for thrombolysis. One is streptokinase, derived from streptococci, which indirectly activates plasminogen and the other is tissue plasminogen activator, a human product which is manufactured by recombinant gene technology. Streptokinase is cheaper but may produce anaphylaxis in patients with previous exposure. The fibrinolytic agent is administered by intra-arterial infusion. After dissolution of the thrombus, the patient will require anticoagulant therapy to prevent further thromboses and any underlying cause for the thrombosis should be treated.

Surgical

Thrombolysis would have little effect on embolic disease. The first line treatment for embolic disease is removal of the embolus. Embolectomy is performed using a balloon embolectomy catheter. This can be used to remove any embolic material from proximal and distal vessels using a single arteriotomy usually in the common femoral artery. As most emboli are composed of thrombus, embolectomy may be supplemented with fibrinolysis to help lyse any distal clot not retrieved by the embolectomy catheter. The cause of the emboli must be actively sought and treated to prevent further episodes.

1 c.ii) TREATMENT OF CHRONIC ISCHAEMIA.

Conservative

Treatment of lower limb vascular disease must involve an holistic approach. Most patients with peripheral vascular disease are unlikely to lose the affected limb (Criado *et al.*1991). They are far more likely to die because of coronary artery atherosclerosis (Smith *et al.*1990, Hertzer 1991, Duprez 1992, Vogt *et al.*1992). This is particularly true when the ABPI is less than 0.5 or the toe pressure less than 40mmHg (O'Riordain *et al.*1991, Dormandy *et al.*1991, Bowers *et al.*1993). This should influence the treatment of the relatively benign

condition of intermittent claudication. Up to 66% patients will have improved symptoms after simple conservative measures such as stopping smoking and exercising through the pain (O'Riordain *et al.* 1991). Treatment of hypercholesterolaemia, when present, can also be expected to have some benefit. If the symptoms do not improve, then the patient needs to consider whether he wishes to risk an operation. In most cases, operative treatment can be expected to improve symptoms. However, it can also worsen them. Patients with critical ischaemia should be offered reconstructive surgery wherever possible (Harling *et al.* 1987, Tyrrell *et al.* 1989, Paetz *et al.* 1991, Sayers *et al.* 1992a, Cheshire *et al.* 1992). Primary amputation can only be justified when reconstruction is impossible or if it is unlikely to benefit the individual patient because of coexisting disease. Whilst reconstructive surgery is often expensive, the overall cost is exceeded by the cost to the community of an amputation (Cheshire *et al.* 1991, Cheshire *et al.* 1992) which in 1992 was estimated to be in excess of £25,000 (Harris 1992).

Endovascular

Angioplasty

Percutaneous Transluminal Angioplasty (PTA) is a relatively simple procedure, first described by Dotter & Judkins in 1964 (Dotter *et al.* 1964). They originally used coaxial dilating catheters to dilate arterial stenoses. The technique was improved by the development of a balloon catheter to dilate the vessel, some 10 years later (Gruntzig *et al.* 1979). Initially the technique was only applicable for stenoses or short occlusions which could easily be crossed by a guidewire.

The number of PTAs performed has increased dramatically over the past 20 years or so (Sayers *et al.* 1993c) and a large number of limbs which would previously have required reconstructive surgery or would have been managed conservatively are now treated with PTA. Several centres have even reported acceptable results for crural vessel angioplasty (Bakal *et al.* 1990, Saab *et al.* 1992, Flueckiger *et al.* 1992, Bolia *et al.* 1994). The initial success rates for peripheral occlusions are in the range of 70-80%, but the patency rate at 1 month is as low as 52% (Jeans *et al.* 1990). Reported success rates at 6 and 12 months have been as low as 22.5% and 13.5% respectively (Feinberg *et al.* 1990).

Even a relatively simple procedure such as PTA is not without complications. Arterial dissection (Korogi *et al.* 1992), thrombosis (Jorgensen *et al.* 1990), embolism, perforation, (Chong *et al.* 1990, Reed *et al.* 1991) arteriovenous fistulae (Borstlap *et al.* 1993) and late aneurysm formation (Vive *et al.* 1992) can all occur. Such complications are relatively rare (O'Keeffe *et al.* 1991, Mahler *et al.* 1992, Hunink *et al.* 1993, Struk *et al.* 1993). Major complications are more common in the elderly (Morse *et al.* 1991) and where the procedure is carried out for critical ischaemia (Johnston 1992, Hunink *et al.* 1993). In a large study of 1642 angioplasties performed in Sheffield (Belli *et al.* 1990) only 0.7% of patients undergoing PTA for intermittent claudication had a significant side effect and only 0.5% required reconstructive surgery.

The advent of laser-assisted balloon angioplasty was thought by some to be an improvement on PTA alone (Salvian *et al.* 1990), as it potentially allowed complete occlusions to be passed by the guidewire prior to PTA. However, early enthusiasm for the technique has waned as laser-assistance has little practical advantage over conventional angioplasty (Owen *et al.* 1990).

Recently subintimal angioplasty, developed in Leicester, has been shown to be useful in treating both short and medium sized arterial occlusions with an acceptable complication rate (Bolia *et al.* 1990).

Stents

Intravascular stent placement may be used either alone or in combination with PTA to maintain the patency of a blood vessel. Stents may be either self-expanding or balloon distensible. The radial pressure exerted by the stent within the vessel is able to maintain the vessel patency and prevent immediate collapse of the vessel after an initially successful angioplasty. Endothelial cells can grow in and form a lining over the stent making the area less thrombogenic. In Palmaz's initial animal work, reendothelialization over stents in porcine renal arteries was complete at 3 weeks (Palmaz *et al.* 1987), though angioscopic studies of human coronary arteries suggest that the time scale may be longer in humans (Ueda *et al.* 1994). The initial clinical results from stenting of iliac artery stenoses were very promising (Palmaz *et al.* 1988) and intravascular stent placement has produced good long term results in large vessels with good blood flow (Palmaz 1992). The treatment of isolated iliac artery stenoses is the most

common use for intravascular stents. There has not been any convincing randomised trial showing benefit for stent deployment in the peripheral circulation although there has been a trial showing benefit for stents in the coronary circulation (Fischman et al.1994).

As with any prosthetic material, intravascular stents may initiate thrombus formation (Do *et al.*1992, van Beusekom *et al.*1994). This is most likely to be a problem in small vessels with poor flow and where the stent is causing an appreciable deformity of the lumen with resulting turbulent flow. Intimal hyperplasia can develop underneath the intact endothelial covering of a stented blood vessel (Do *et al.*1992, Sapoval *et al.*1992). This can be difficult to treat with endovascular techniques as it is not usually possible to distend a stent further with balloon angioplasty and once in place, stents are difficult to remove.

Atherectomy

Angioplasty and intravascular stenting both compress atherosclerotic material within the vessel lumen against the wall of the vessel. In theory, removal of the atherosclerotic debris should offer advantages over compression. Transluminal atherectomy allows debulking and also provides tissue for histological examination, confirming that restenotic lesions after previous intervention are due to intimal hyperplasia (Johnson 1990, Barbieri *et al.*1991, Garratt *et al.*1991). Several authors have reported good early results with this technique (Graor *et al.*1990, Wilms *et al.*1990). However, other authors have suggested that the results are no better than those from conventional PTA (Kotb *et al.*1992) and there is a high restenosis rate after atherectomy (Ahn 1992a, Ahn *et al.*1992b). The process of mechanical atherectomy appears to have a potent stimulatory effect on intimal hyperplasia. It may have a limited application in treating restenoses in previously stented arteries where PTA can not adequately dilate the stented vessel (Vorwerk *et al.*1990). In such cases, mechanical atherectomy is able to remove the neointima which forms on the luminal surface of the stent.

Surgical

Endarterectomy

Open endarterectomy is the treatment of choice for symptomatic stenotic internal carotid arteries but is rarely carried out in the lower limb. This is probably due to the increased

use of PTA over the past 20 years or so. Endarterectomy of short occlusions of the aortoiliac segment can produce good results (Naylor *et al.* 1990) but there is a high recurrence rate if the patient continues to smoke, particularly if the external iliac artery is affected. Open endarterectomy of the superficial femoral artery can also produce good results with 5 year patencies of up to 71% in experienced hands (van der Heijden et al. 1992).

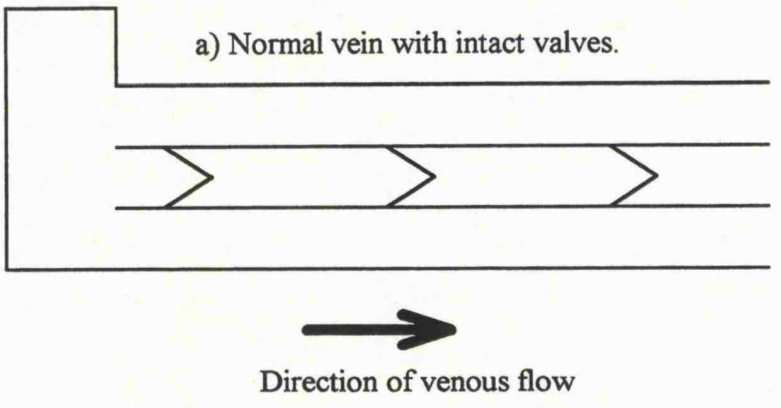
Arterial bypass surgery

The classical surgical treatment for peripheral vascular disease is bypass surgery. The diseased segment of vessel is bypassed so that there is a patent channel between the inflow vessel and the run-off vessel. Autologous saphenous vein is the conduit of choice for peripheral bypass surgery (Budd *et al.* 1990) but it is not always available. There are a whole range of synthetic and biological grafts available. The most popular synthetic material for infrainguinal bypasses is expanded polytetrafluoroethylene (PTFE), followed by dacron. The 2 common biological materials available are tanned Human Umbilical Vein and bovine vein.

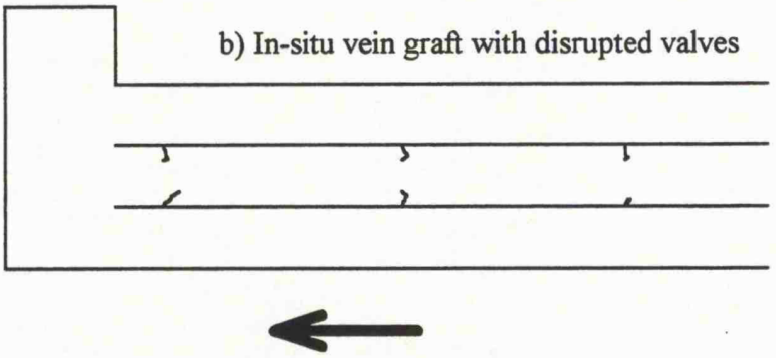
Vein grafts

Autologous vein, when available, can either be reversed or it can be left in situ and the valves can be destroyed using a valvulotome. Autologous vein has the advantage that it is the patient's own vascular tissue which is being used to transport blood, albeit at higher pressure than usual. Veins are not as elastic or as muscular as arteries and the grafted vein is not able to contract or recoil to the same extent as an artery. Veins also differ from arteries in that they have valves to direct blood flow towards the heart. As arteries direct blood away from the heart, the vein must either be reversed or the valves have to be rendered incompetent. Figure 1c.1 is a diagrammatic representation of a normal vein, a reversed vein graft and an in-situ vein graft. The use of autologous vein (reversed) for arterial bypass surgery was first described by Kunlin in 1949 (Kunlin 1949) and a technique for in situ vein graft bypass by excising the valves was first described by Hall in 1962 (Hall 1962). The long saphenous vein is usually chosen for arterial bypass surgery as this vein is usually of adequate length and diameter for most arterial bypass procedures in the lower limb and its removal from the venous system does not significantly impair venous return in the lower limb. Other veins which are frequently used

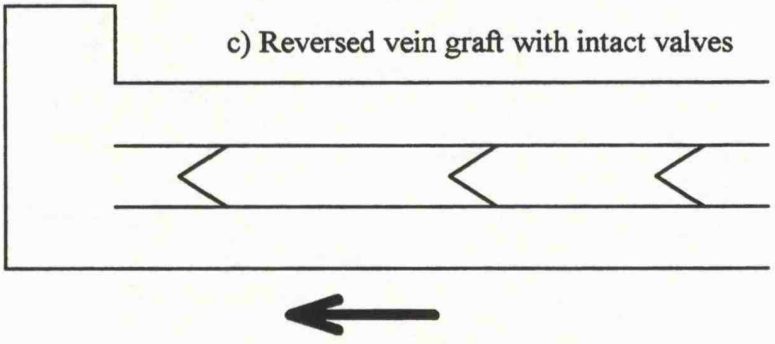
Figure 1c.1 Diagrammatic representation of the venous valves a) in their normal position, b) after valvulotomy for an in-situ bypass graft and c) in a reversed vein graft.



Direction of venous flow



Direction of arterial flow

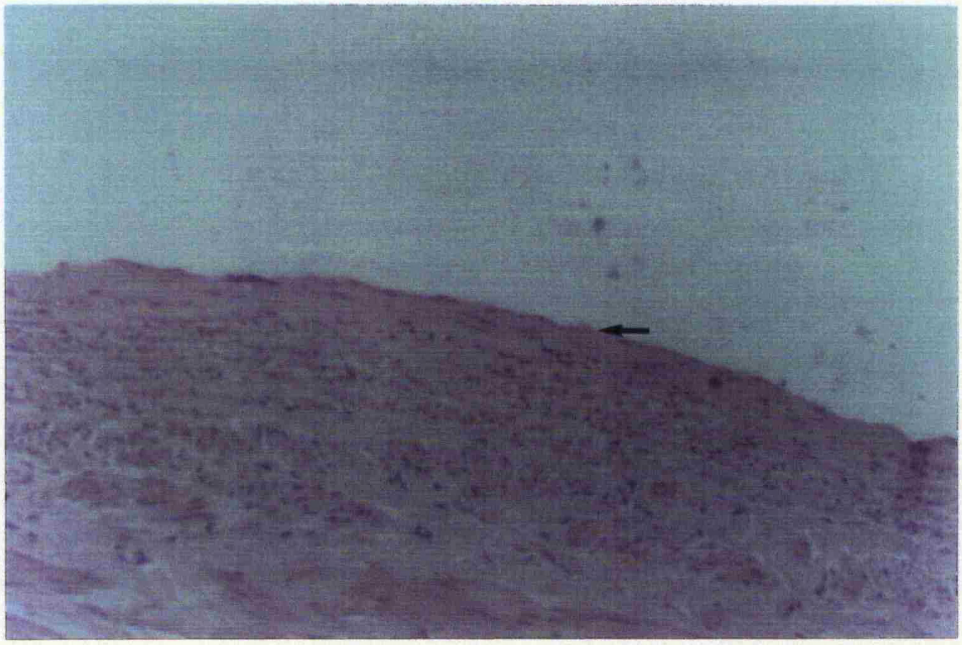


Direction of arterial flow

include the short saphenous vein in the calf, and the cephalic and basilic veins from the forearm (Chalmers *et al.* 1994a).

Classically, human vein has a thin intima, a relatively thin media containing some smooth muscle cells and connective tissue with a thin adventitial layer. However, microscopic examination of human saphenous vein, removed for use in peripheral or coronary artery bypass surgery shows that many veins have significant pre-existing abnormalities (Davies *et al.* 1993a, Panetta *et al.* 1992b). There is often some thickening of either the media or intima in macroscopically normal veins. It has been suggested that this pre-existing disease may contribute to subsequent graft failure and that pre-operative screening by measurement of vein compliance may be of benefit (Davies *et al.* 1992) in recognising abnormal vein before it is used.

Figure 1c.2 Macroscopically 'normal' vein harvested for use in peripheral bypass surgery x 100 (Haemotoxylin + Eosin). Note the relatively thick intima (arrow).



The in situ method of bypass grafting is preferred by some individuals as the vein is not disturbed and the distal part of the vein is a closer size match to the distal artery, especially when the graft extends below the knee. This is thought to improve the overall long term patency (Leather *et al.* 1981, Fogle *et al.* 1987). There is minimal dissection of the vein as it does not have to be removed and reversed. This helps retain the vasa vasorum intact which may be important in inhibiting intimal hyperplasia (Buchbinder *et al.* 1981, Barker *et al.* 1993, Martin *et al.* 1990, Martin *et al.* 1991). It is also possible to limit dissection of the lower limb as it is only necessary to make incisions for the upper and lower anastomoses and to ligate side branches which would cause arteriovenous fistulae. These side branches can be located by arteriography at the time of operation, by duplex ultrasound preoperatively or by angioscopy. The relatively new technique of angioscopy allows localisation of side branches peroperatively (Maini *et al.* 1993, Davies *et al.* 1993b) and can be used to treat them by coil embolisation. This further reduces the 'wound length' for the patient and can mean that only 2 incisions are required, one at the upper and one at the lower anastomosis (Cikrit *et al.* 1994). Angioscopy provides useful information about venous anatomy in up to 85% of vein grafts (Grundfest *et al.* 1988). It is able to demonstrate sclerotic or otherwise abnormal vein (Sales *et al.* 1993) and its use to guide valve destruction under direct vision can help reduce valvulotome injury as well as ensuring that fewer residual competent valves are left behind (Miller *et al.* 1991). Although most reports of angioscopy are enthusiastic, there are few randomised trials. One reported randomised trial of the technique showed no significant difference in wound morbidity, length of hospital stay or midterm graft patency (Clair *et al.* 1994).

Advocates of the in-situ technique argue that it is less traumatic than the reversed vein method. However, it is often necessary to translocate the vessel in order to reach a distal artery so that the vein is not really 'in-situ'. It is merely 'not reversed' and still has to be dissected free of surrounding tissue, destroying any vasa vasorum that may be present. Also it has been demonstrated in our department that the technique of valve destruction causes significant injury to the vein (Sayers *et al.* 1991, Sayers *et al.* 1992b) with complete loss of the endothelial layer and patchy necrosis of SMCs in the media.

Damage to reversed vein grafts may be caused by uncontrolled distension during surgical preparation of the vein. This can be demonstrated in the laboratory by a fall in the ATP/ADP ratio (Angelini *et al.* 1985), or by decreased prostacyclin (Angelini *et al.* 1987), or Endothelium-Derived Relaxing Factor (EDRF) production (Angelini *et al.* 1989). This damage can be largely avoided if the vein is distended in a controlled manner at a pressure of less than 300mmHg (Angelini *et al.* 1985), or if distended by the patient's own arterial pressure (Angelini *et al.* 1992a). The reversed saphenous vein has to be dissected free from the surrounding tissues. All tributaries have to be ligated and divided so that the vein can be reversed. This reduces the incidence of arteriovenous fistulae markedly compared to the in-situ technique as the reversed vein should no longer be connected to the venous system. The dissection of the vein free from all its surrounding tissue will, by its very nature, destroy the vasa vasorum supplying the vein wall. Destruction of the vasa vasorum in rabbit arteries has been shown to initiate intimal hyperplasia (Barker *et al.* 1993, Martin *et al.* 1990, Martin *et al.* 1991) although the relevance of this to human saphenous vein is uncertain. It is technically difficult to reduce the length of the incision with reversed vein grafts as the whole of the vein must be mobilised so that it can be removed and reversed for use as a bypass conduit. This usually involves one long incision over the entire length of the vein to be excised, though some surgeons prefer to make several short interrupted incisions over the vein.

The in-situ and reversed methods of bypass grafting with vein both have their advocates. Each method has its own advantages and disadvantages. Most surgeons tend to use the in-situ method for long distal bypasses where the size of the small crural or pedal vessel is a closer size match to the distal saphenous vein. However, the use of reversed or in-situ vein in more proximal grafts is very much a matter of personal preference. A prospective randomised controlled trial has failed to show any significant difference in patency between in situ and reversed grafts (Harris *et al.* 1993, Wengerter *et al.* 1991).

Surveillance

Vein grafts have a better long-term patency rate than any currently available synthetic graft (Budd *et al.* 1990). In order to standardise reporting of graft patency rates between centres, the Society for Vascular Surgery suggested that: uninterrupted patency should be

referred to as *primary patency*; uninterrupted patency with a procedure, such as PTA, performed to maintain patency should be referred to as *primary assisted patency*; and patency following a salvage procedure for graft occlusion should be referred to as *secondary patency* (Rutherford 1991). Most surgeons quote secondary patency rates of between 65-100% at 1 year (DeWeese *et al.* 1977, Buchbinder *et al.* 1981, Levine *et al.* 1985, Leather *et al.* 1988, Bejcek *et al.* 1989, Berkowitz *et al.* 1989, Hickey *et al.* 1991, Bergamini *et al.* 1991), and between 59-82% at 5 years (DeWeese *et al.* 1977, Leather *et al.* 1988, Berkowitz *et al.* 1989, Taylor *et al.* 1990, Bergamini *et al.* 1991, Hickey *et al.* 1991). Primary patency rates are not always quoted but are invariably lower. Reported primary patency rates at 1 year vary from 64-85% (Wengerter *et al.* 1991, Moody *et al.* 1992b) with 5 year rates between 41-75% (Budd *et al.* 1990, Taylor *et al.* 1990, Moody *et al.* 1992b). Some of the differences in the patency rates between centres are due to differences in the numbers of distal bypasses and differences in the proportion of patients presenting with claudication or critical ischaemia. Grafts to more distal vessels and bypasses for critical ischaemia fare worse than those to more proximal vessels and those for intermittent claudication.

Vein graft failure in the first 30 days is usually due to 'technical error' (Wolfe *et al.* 1987). This may mean a surgical error, leading to kinking of the graft or narrowing of one or both anastomoses or there may be an error in patient or operation selection. A graft on to an occluded or a severely diseased vessel is unlikely to work if the flow rate through the graft is inadequate to prevent thrombosis. Such 'technical errors' can hopefully be avoided if appropriate preoperative investigations have been performed (Bell 1991). A multivariate analysis of risk factors involved in early vein graft occlusion in our department found that a high peripheral resistance (>1.4 Peripheral Resistance Units) was the only significant independent risk factor (Varty *et al.* 1993b) which could be used to predict early graft failure.

After the first month, the commonest cause of vein graft failure is the development of vein graft stenoses (McNamara *et al.* 1967, Whitney *et al.* 1976, Berkowitz *et al.* 1992, Mills *et al.* 1993). These stenoses are due to intimal hyperplasia (Sayers *et al.* 1993a) and tend to occur at focal points in the graft. Traditionally, these stenoses were considered as occurring as a 'response to injury' and were thought to occur at sites of clamp injury or at sites of damaged

venous valves (McNamara *et al.* 1967, Szilagyi *et al.* 1973). However, recent studies have shown that stenoses do not develop at sites of vein injury, or at tributary or venotomy sites. (Moody *et al.* 1992a, Davies *et al.* 1994). There is some evidence that early duplex scanning of vein grafts at about 3 months after surgery may be able to detect flow abnormalities with a high probability of developing into graft stenoses (Mills et al. 1995). If these results are confirmed by other workers, then early scanning may yield more information on the aetiology of vein graft stenoses. Histological examination of stenoses shows them to consist of proliferating vascular smooth muscle cells in an extracellular matrix (Sayers *et al.* 1993a). These stenoses are initially asymptomatic but will go on to cause vein graft occlusion as they progressively narrow the lumen of the vessel (London *et al.* 1993). Once a vein graft has occluded, the results of any salvage procedures performed on the graft are poor (Belkin *et al.* 1990). Therefore, if it is possible to prevent stenoses from progressing to occlusion, overall vein graft patency should be improved. The purpose of a vein graft surveillance programme is to detect and treat such stenoses whilst they are still asymptomatic.

Surveillance programmes for infrainguinal vein grafts have been shown to be of benefit in improving graft patency in several studies (Berkowitz *et al.* 1981, Grigg *et al.* 1988a, Disselhoff *et al.* 1989, Moody *et al.* 1990, Mills *et al.* 1990, Brennan *et al.* 1991, Berkowitz *et al.* 1992, Harris 1992, Idu *et al.* 1992, London *et al.* 1993, Mills *et al.* 1993, Sayers *et al.* 1993b). The essence of a vein graft surveillance programme is the use of non-invasive methods to detect stenoses before they become symptomatic. Serial angiography is able to detect stenoses but is invasive, expensive and not without side effects. The non-invasive procedures commonly used are serial measurements of ankle-brachial pressure indices (ABPI) and Doppler Ultrasound or *Duplex* scanning. These techniques are demonstrated in Figures 1c.3 and 1c.4. The advantage of duplex scanning over normal B-mode ultrasound is that it also has the capacity to measure changes in velocity of blood flow which occur at sites of stenosis. There is an increase in peak velocity at the site of a significant stenosis in comparison to the segment of vessel 2cm proximal to the stenosis, and there is a generalised fall in the peak velocity throughout the graft (Bund *et al.* 1989). Most centres look for a peak segmental increase in velocity of 2-3 times at a stenosis and/or a fall in velocity to ~45cm/s (Taylor *et al.* 1992, Bandyk *et al.* 1985). Figure 1c.5 shows a colour duplex image of a normal vein graft along its

entire length. Figure 1c.6 shows a colour duplex scan of a vein graft stenosis whilst Figure 1c.7 shows the velocity changes seen with duplex at the site of a stenosis. Serial measurement of ankle brachial pressure indices is a useful screening method for vein grafts (Berkowitz *et al.*1981). A serial fall in the ABPI >0.1 is usually taken as being indicative of a significant flow disturbance. A post-exercise fall in ABPI is of even greater predictive value when assessing vein grafts and may be used as a screening tool before duplex scanning (Brennan *et al.*1991). The combination of ABPI measurement and duplex scanning has a predictive value as good as more invasive methods such as digital subtraction angiography (Moody *et al.*1990, McShane *et al.*1989, Grigg *et al.*1988a, Disselhoff *et al.*1989, Londrey *et al.*1990).

Figure 1c.3 Measurement of ankle brachial pressure indices offer a quick and reliable method of assessing vein grafts.

Figure 1c.4 A duplex ultrasound scan is able to give information on the position and haemodynamic significance of vein graft stenoses. In the hands of a trained operator, this technique can provide quick and accurate information about the vein graft.

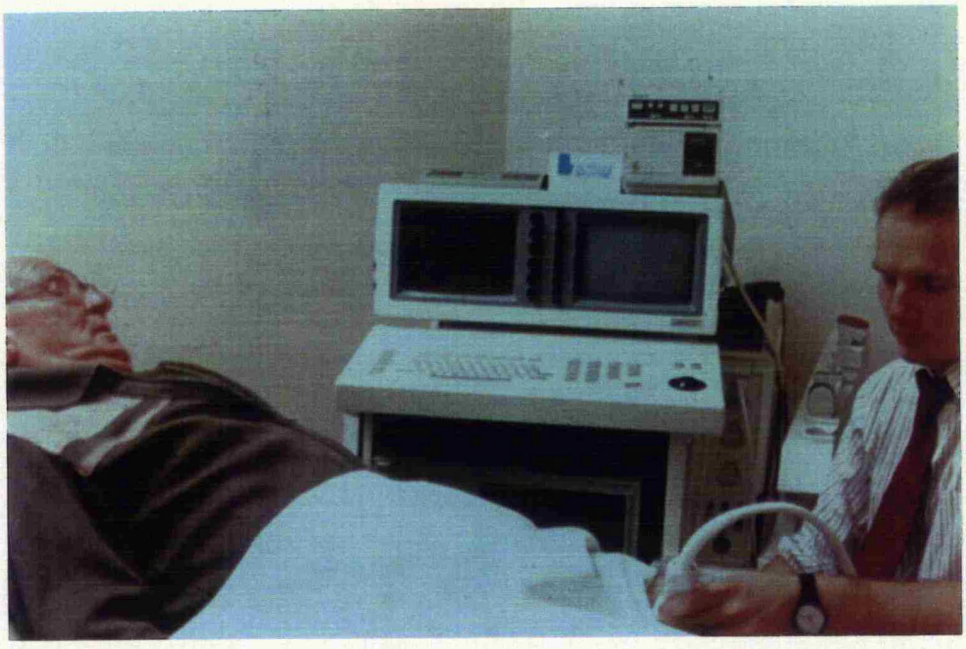


Figure 1c.5 Composite picture of a colour duplex scan of a vein graft showing the entire length of the graft.

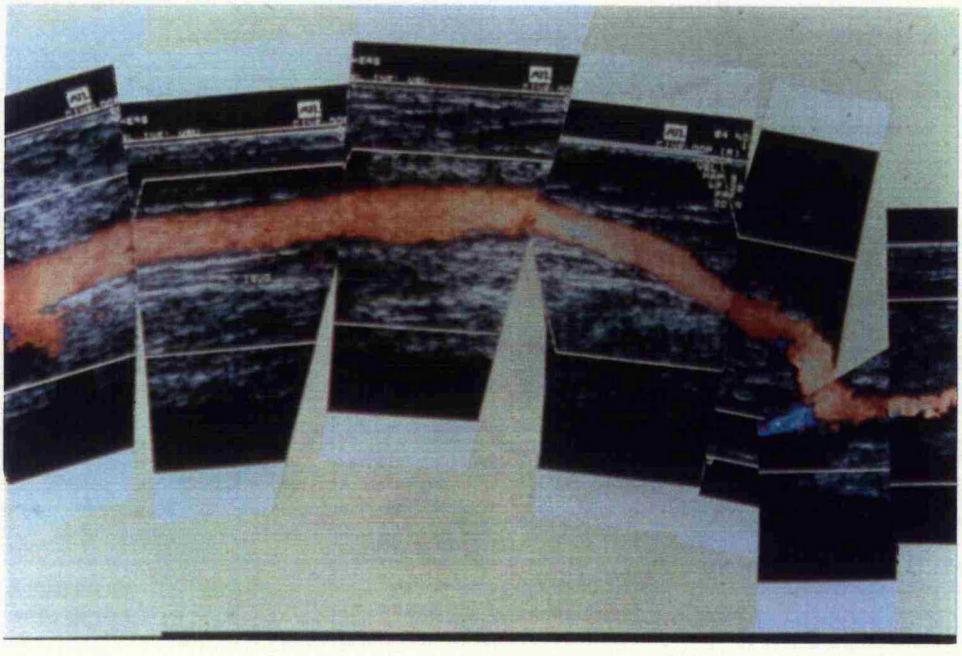


Figure 1c.6 Colour duplex scan of a vein graft showing a stenosis.

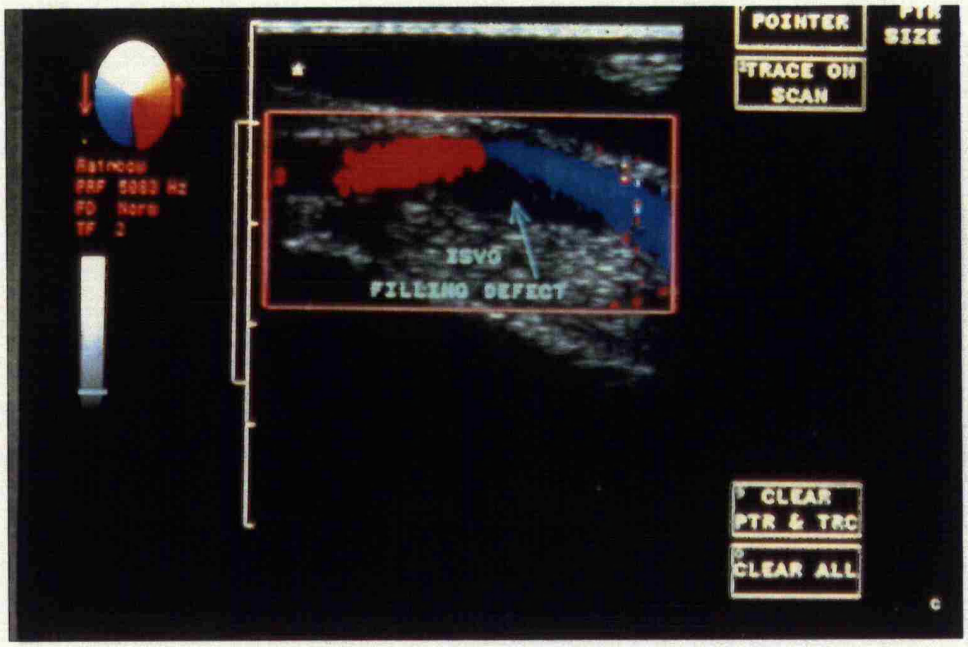
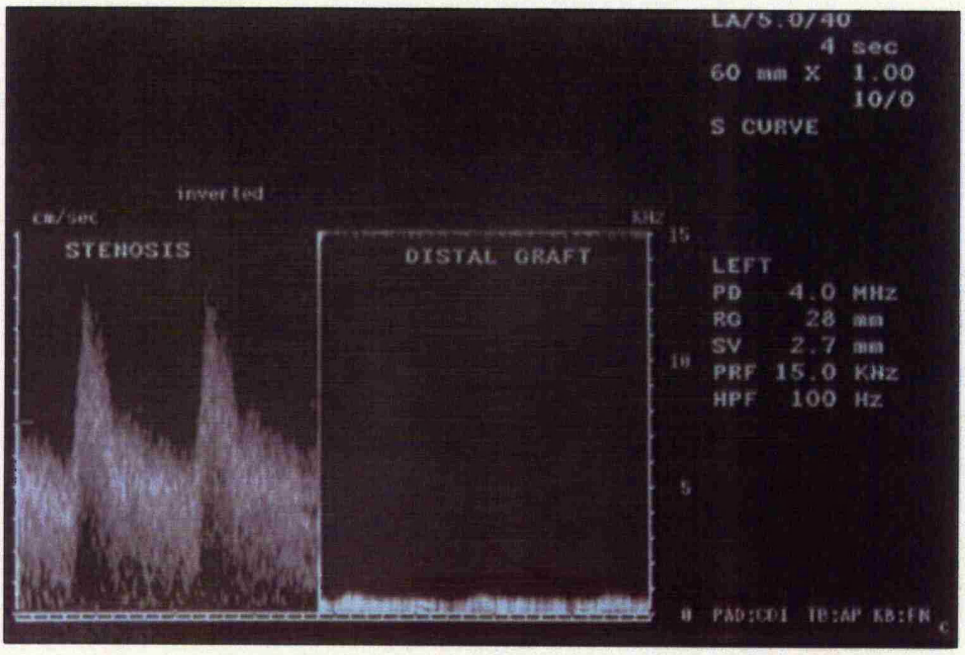


Figure 1c.7 Doppler spectrum at vein graft stenosis using duplex scan. There is a velocity increase at the stenosis with a fall in velocity beyond the stenosis.



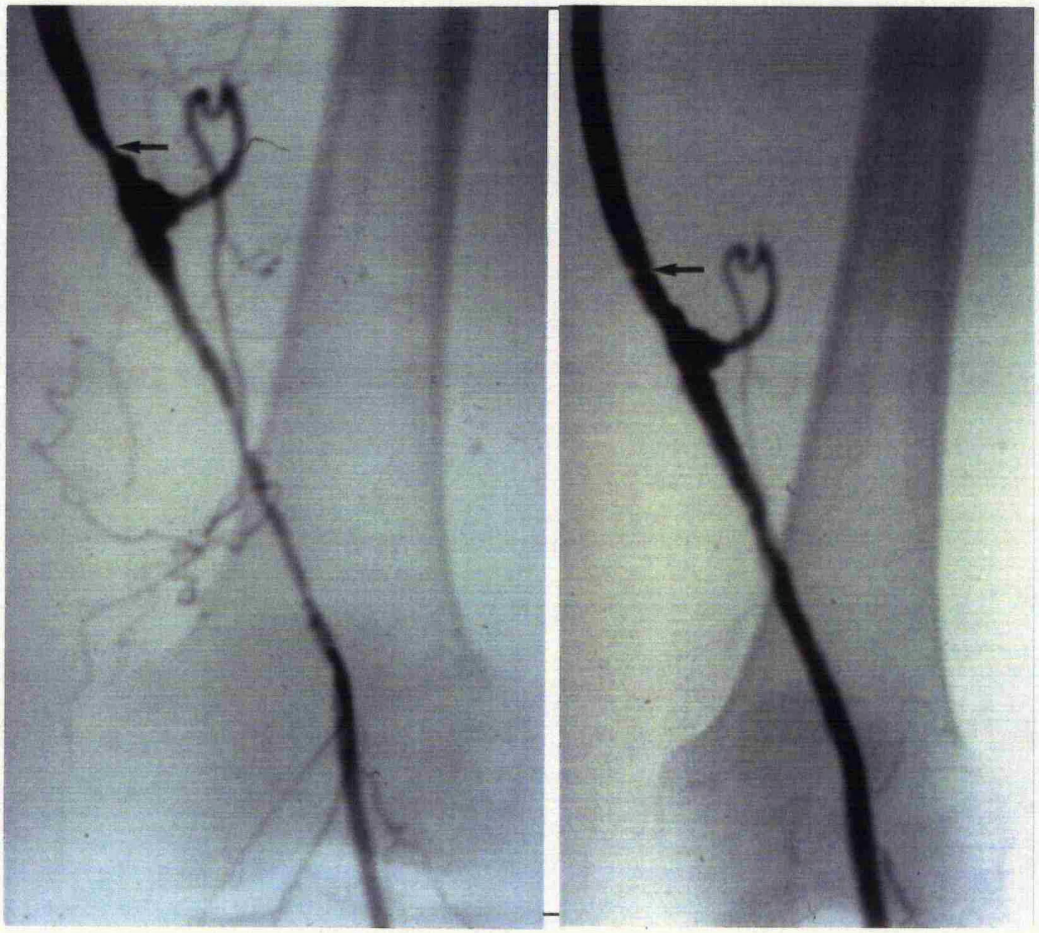
Treatment of vein graft stenoses

Vein graft stenoses are caused by a focal proliferation of vascular smooth muscle cells with production of extracellular matrix, a process known as intimal hyperplasia. Stenoses occur in 20-30% of all vein grafts (Szilagyi *et al.* 1973, Sladen *et al.* 1981, Grigg *et al.* 1988a, Moody *et al.* 1989, Moody *et al.* 1992a) and are therefore a common problem. They were first recognised in the 1960's (Breslau *et al.* 1965, McNamara *et al.* 1967) although it wasn't until the report by Szilagyi et al. (Szilagyi *et al.* 1973) that their true incidence and significance for vascular surgery was realised. Although it has been argued that not all stenoses will go on to cause graft occlusion (Moody *et al.* 1989), a significant number will occlude (London *et al.* 1993) and it is therefore important to treat them promptly.

The simplest method and the first line treatment in most centres is Percutaneous Transluminal Angioplasty (PTA). This is the same technique used to dilate atherosclerotic plaques in diseased arteries. Its first reported use in treating vein graft stenoses was by Alpert et al. in 1979 (Alpert *et al.* 1979). The technique is relatively atraumatic with a low complication rate (Belli *et al.* 1990, Belli 1991). It is also possible to carry out the technique under local anaesthetic as a day case without the inherent risks of general anaesthesia. Most stenoses are suitable for treatment with PTA (London *et al.* 1993) but some individuals have doubted whether it is as effective as operative graft revision (Thompson *et al.* 1989, Perler *et al.* 1990, Whittemore *et al.* 1991) and recommend its use only for short (<2cm long) stenoses. Berkowitz et al. found that those lesions in the mid-graft and those at the distal anastomosis did particularly badly after angioplasty (Berkowitz *et al.* 1992) and recommended that these stenoses and all recurrent stenoses should be treated by open revision. One experiment involving the *in vitro* angioplasty of human vein graft stenoses removed at operation showed that lesions with marked intimal thickening showed little improvement in diameter after PTA (Marin *et al.* 1993a). This report also showed a higher incidence of intimal flaps post angioplasty in those stenoses with a high degree of cellularity, suggesting that older, more established stenoses probably do less well after PTA. The results of PTA in our own department are given in Chapter 4.

There is limited experience of intravascular stents in the management of vein graft stenoses (Davies *et al.* 1993a). Clinical and angiographic studies of stenting of coronary artery bypass saphenous vein grafts have shown a trend towards improved patency compared to PTA alone (Strumpf *et al.* 1992). Animal models of vein graft stenoses suggest that a single stent can provide as good a patency as PTA, but multiple stents lead to a lower long-term patency (van Beusekom *et al.* 1994).

Figure 1c.8 Angiogram of vein graft stenosis (arrow) before and after PTA. There is still some residual stenosis of the vein post PTA.



At present, stenoses have to be treated after they have occurred. Ideally, prevention of stenoses before they occur would be preferable. Careful surgical technique with minimal trauma to the vein and avoidance of high distension pressures may help prevent stenoses by

limiting the initial injury to the vein. However, no controlled trials have been done to prove that minimally damaged vein develops fewer stenoses, and Moody et al. were not able to link the development of graft stenoses to any area of known vein damage (Moody *et al.* 1992a). Pharmacological control of intimal hyperplasia is a possibility. Antiplatelet drugs, calcium antagonists, lipid-lowering drugs, fish oil, heparin, angiotensin-converting enzyme inhibitors, cyclosporin, prazosin and steroids can all reduce intimal hyperplasia in various animal models (Varty *et al.* 1993a) but no drug has been convincingly shown to alter the course of intimal hyperplasia in humans (Clowes 1991a). There has been a recent trial which showed an improvement in patients taking low molecular weight heparin compared to aspirin and dipyridamole with a suggestion by the authors that the heparin had inhibited the intimal hyperplasia (Edmondson *et al.* 1994). However, this work had several methodological errors (London *et al.* 1994). All patients received heparin at the time of operation, which is when any antiproliferative effect might be expected to take place, and no improvement was seen in patients with intermittent claudication. Graft patency was improved in those patients with critical ischaemia but there was no evidence that this improvement was due to a reduction in intimal hyperplasia.

Advances in molecular biology offer another possible means of preventing stenoses. It is possible to apply antisense oligonucleotides in a gel onto the adventitia of a blood vessel and reduce subsequent intimal hyperplasia. This has been done in animal models with antisense oligonucleotides to both the c-myb (Simons *et al.* 1992a) and c-myc (Bennett *et al.* 1994) protooncogenes. It was possible to inhibit arterial smooth muscle cell proliferation in animals following PTA using this technique, but whether this can be applied to venous smooth muscle cells in humans remains to be shown.

Synthetic grafts

Autologous saphenous vein is the best available conduit for arterial bypass surgery in the lower limb (Budd *et al.* 1990). When the long saphenous vein is not available, it is worth resorting to the short saphenous vein or arm veins which can be used alone or in a composite graft. These alternative sources of vein can produce quite acceptable results (Taylor *et al.* 1987, Hickey *et al.* 1991, Sayers *et al.* 1993b). If there is no vein available, then synthetic or

alternative biological material can be used. The most popular synthetic materials in use for infrainguinal bypass surgery are expanded Polytetrafluoroethylene (PTFE) and dacron.

PTFE is the most widely used synthetic material for infrainguinal grafts. It was first reported as being used as a blood vessel prosthesis in the early 1970's in animal models (Soyer *et al.* 1972, Campbell *et al.* 1974, Campbell *et al.* 1975). Campbell et al. also presented the first clinical series of PTFE being used as an arterial substitute in 15 patients with critical ischaemia and no available vein (Campbell *et al.* 1976). The early results were quite promising with an initial limb salvage rate of 87% in this small series. However, contemporary results were not always as impressive. Clyne et al. reported a 50% graft patency at 6 months (Clyne *et al.* 1979), and Kidson et al. reported a 52% patency at 1 year (Kidson *et al.* 1981). Since the mid-1980s, most authors have reported patency rates at 1 year of between 40-90% (Williams *et al.* 1985, Charlesworth *et al.* 1985, Taylor *et al.* 1987, Prendiville *et al.* 1990, O'Riordain *et al.* 1992). However, the long term results of PTFE do not match up to those of autologous vein with 5 year patency rates of between 18-60% (Williams *et al.* 1985, Charlesworth *et al.* 1985, Taylor *et al.* 1987, Budd *et al.* 1990, Prendiville *et al.* 1990, O'Riordain *et al.* 1992). The best reported 5 year patency rate for above-knee popliteal bypasses is 63% (Davies *et al.* 1991).

PTFE grafts do not develop intrinsic stenoses, although anastomotic stenoses due to intimal hyperplasia do occur (Clowes *et al.* 1986). The principal cause of PTFE graft failure is progression of atherosclerosis in both run off and inflow vessels (Veith *et al.* 1980, O'Donnell *et al.* 1984, Sterpetti *et al.* 1985, Taylor *et al.* 1987, Quinones-Baldrich *et al.* 1991). Anastomotic intimal hyperplasia (Clowes *et al.* 1986) and 'technical failures' are the next most common causes of graft failure. As synthetic materials are more thrombogenic than native vessels, graft occlusion is more likely to occur if there is a decrease in blood flow due to progression of atherosclerosis or an anastomotic stenosis. The mechanism of intimal hyperplasia in PTFE grafts is similar to that in native vessels. There is an ingrowth of endothelial and smooth muscle cells (SMCs) into the prosthetic graft (Clowes *et al.* 1986) with proliferation of the SMCs underneath the endothelial layer. This proliferation is most marked in humans at the distal anastomosis (Taylor *et al.* 1987, Chalmers *et al.* 1994b). It is thought that

compliance mismatch between the synthetic graft and the native artery may be partly responsible for the hyperplasia at the distal anastomosis.

The compliance mismatch can be improved by incorporating a piece of saphenous vein at the distal anastomosis, either as a patch or as a complete cuff. A vein cuff was first described as a technique to facilitate the anastomosis of vein to dacron or diseased artery (Siegman 1979). A modification of this method by Miller et al. was used to improve the anastomosis of a PTFE graft to a distal artery (Miller *et al.* 1984). This made the anastomosis of the relatively inflexible prosthetic graft to the friable artery much easier. Miller also commented that the procedure may have a beneficial effect on the development of intimal hyperplasia. An *in vitro* model of this interposition vein cuff has shown haemodynamic advantage compared to direct anastomosis of PTFE to artery (Beard *et al.* 1986). An anastomotic vein patch has been used by Taylor et al. with good 5 year patency rates up to 71% for popliteal and 54% for infrapopliteal grafts (Taylor *et al.* 1992). *In vitro* experiments comparing the vein cuff, a vein patch and direct PTFE to artery anastomoses have also shown a haemodynamic benefit from the use of vein (Tyrrell *et al.* 1990, Tyrrell *et al.* 1992). A multicentre randomised trial under the auspices of the Joint Vascular Research Group of the Vascular Surgical Society was commenced in 1991 to compare the clinical outcome of PTFE grafts with and without an interposition vein cuff. This showed no significant difference at 12 or 24 months for grafts to the above-knee popliteal artery, but there was a significant improvement in graft patency and limb salvage where the distal anastomosis was to an infragenicular vessel.

Graft surveillance for vein grafts is of proven benefit in maintaining graft patency. However, the benefits from surveillance of PTFE grafts are not so clear. As the main causes of PTFE graft failure are progression of atherosclerosis and anastomotic intimal hyperplasia, any surveillance programme would have to detect and allow treatment of these problems before graft occlusion occurred (Sanchez *et al.* 1993a). As the results of treating a failed graft are worse than treating a failing graft, a surveillance programme would be of benefit in improving graft patency if it was cost effective and detected enough treatable lesions (Sanchez *et al.* 1993b). Stenoses can be treated by open operation or by PTA and inflow and run-off disease can be treated by PTA or by further arterial bypass surgery. If the graft has already

occluded, then it may be treated by thrombolysis or thrombectomy prior to treating the underlying cause (Veith *et al.* 1980) but the results are not as good. One report of a prospective surveillance programme of 69 PTFE grafts suggests that surveillance is of little benefit in improving overall graft patency (Lalak *et al.* 1994). Only 4 grafts developed treatable lesions over a 4 year period and 27 grafts occluded without any warning.

The fact that PTFE is still widely used is a testament to the fact that no new graft has been convincingly shown to be superior. Dacron, polyurethane, and bovine vein have all been used but have no proven advantages over PTFE. Human umbilical vein (HUV) has produced slightly (but not significantly) better secondary patency results in one multicentre randomised trial (McCollum *et al.* 1991). However, the primary patency was not significantly better than that of PTFE, and HUV runs the risk of developing aneurysmal dilatation with time.

1 d) CURRENT CONCEPTS OF VEIN GRAFT STENOSIS.

As mentioned above, vein graft stenoses represent the commonest cause of vein graft failure between one month and one year after surgery. The reasons for the development of these discrete stenoses remain cryptic. Whilst much is now understood of the biology of intimal hyperplasia, the occurrence of focal stenoses within vein grafts is an enigma.

Vein graft stenoses are focal areas of excessive intimal hyperplasia on a background of generalised venous intimal and medial thickening (so-called "arterialisation"). It seems logical to deduce that these localised areas of intimal thickening are related to local trauma or flow disturbances. Certainly, endothelial damage (Chervu *et al.* 1990) and altered shear stress (Kraiss *et al.* 1991) are known to be stimulants of vascular smooth muscle cell proliferation. However, several authors have shown no relationship between areas of vein damage or sites of venous tributaries and subsequent stenosis development (Moody *et al.* 1992a, Davies *et al.* 1994). In addition, work from our Department has shown that in situ vein grafts sustain more damage to the endothelium (Sayers *et al.* 1992b) and to the media (Sayers *et al.* 1991), but there is no difference in the incidence of graft stenoses between the two types of vein graft (Cambria *et al.* 1987).

Vein graft stenoses are by no means universal. Only some 20-30% of bypass grafts will develop stenoses (Szilagyi *et al.* 1973, Grigg *et al.* 1988b, Moody *et al.* 1989, London *et al.* 1993). It has been suggested that those patients who do develop such stenoses may be more susceptible because their SMCs are biologically different. Chan et al have shown that the SMCs of patients who develop vein graft stenoses are more resistant to the antiproliferative effects of heparin (Chan *et al.* 1993). They conclude that these patients represent a group who are at high risk of graft stenosis. Other workers have suggested that pre-existing intimal thickening and low vein compliance are risk factors for later graft stenosis (Davies *et al.* 1992). However, neither of these theories explains the focal nature of vein graft stenoses. An inherent resistance to the antiproliferative effects of heparin, or low vein compliance might be expected to lead to a generalised intimal thickening but do not account for the development of discrete stenoses.

The recently published work by Mills et al. on the aetiology of vein graft stenoses (Mills *et al.* 1995) is interesting as it suggests that potential stenoses may be present at a relatively early stage after bypass surgery. They found 44 flow anomalies within 3 months of surgery, most of these during the first 6 weeks. Subsequent graft surveillance showed that 14 of these lesions regressed, 10 remained present but did not progress and 20 progressed further, becoming haemodynamically significant vein graft stenoses. This work is important as it suggests that stenoses can be detected at an early stage and that not all potential stenoses will progress into fully blown stenoses. These areas of flow disturbance may enhance the intimal hyperplastic response leading to enlargement of the stenoses with further flow disturbance. This could lead to a 'vicious circle' with initial small graft stenoses being self-perpetuating. Further investigation of this finding may prove useful in determining the aetiology of vein graft stenoses.

CHAPTER 2 - The Vascular Biology of Intimal Hyperplasia

2 a) INTRODUCTION

2 b) CELLS INVOLVED IN INTIMAL HYPERPLASIA

2 c) GROWTH FACTORS INVOLVED IN INTIMAL HYPERPLASIA

i) PLATELET-DERIVED GROWTH FACTOR

ii) BASIC FIBROBLAST GROWTH FACTOR

iii) TRANSFORMING GROWTH FACTOR BETA

iv) TUMOUR NECROSIS FACTOR ALPHA

v) INTERLEUKIN-1

vi) INSULIN-LIKE GROWTH FACTOR-1

vii) ENDOTHELIN

viii) EPIDERMAL GROWTH FACTOR

2 d) THE EFFECT OF FLOW ON INTIMAL HYPERPLASIA

i) THE EFFECT OF CYCLIC STRAIN ON INTIMAL HYPERPLASIA

ii) THE EFFECT OF SHEAR STRESS ON INTIMAL HYPERPLASIA

2 e) TREATMENT AND PREVENTION OF INTIMAL HYPERPLASIA

i) PHARMACOLOGICAL MANIPULATION OF INTIMAL HYPERPLASIA

ii) MOLECULAR APPROACHES

2 f) CONCLUSIONS

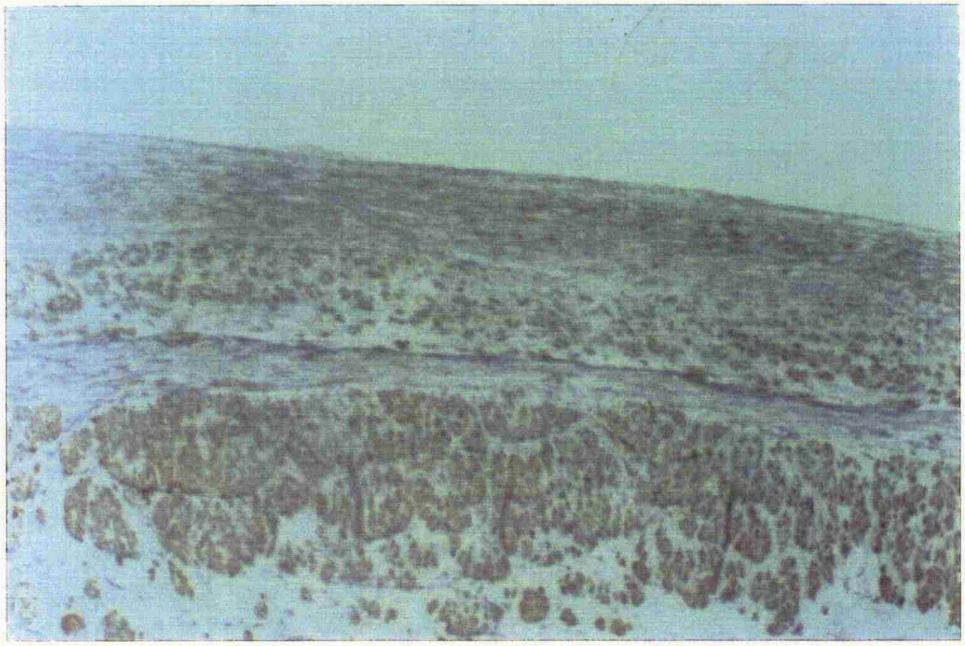
2 a) INTRODUCTION

Intimal hyperplasia is a pathological process affecting blood vessels. There is proliferation of vascular smooth muscle cells (SMCs) with phenotypic change and production of extracellular matrix in the subendothelial layer of the blood vessel concerned. As SMCs are rarely found in the normal intima of blood vessels, there is usually some migration of SMCs from the media of the vessel or from adjoining vessels. The process can occur in arteries, veins or in prosthetic grafts. It is regarded as a 'response to injury' to vascular endothelium (Chervu *et al.* 1990, Painter 1991b, Williams 1991, O'Malley 1992) and is seen following reconstructive vascular surgery (Chervu *et al.* 1990, Cox *et al.* 1991, Varty *et al.* 1993a), angioplasty (Ferns *et al.* 1992, O'Keeffe *et al.* 1991), endarterectomy (Gagne *et al.* 1991, Atnip *et al.* 1990, Graor *et al.* 1990) and organ transplantation (Davies *et al.* 1989, Yilmaz *et al.* 1992). Intimal hyperplasia is an initiating event in atherosclerosis and is the main cause of vein graft stenoses which account for most vein graft failures between one month and one year after arterial bypass surgery (Wolfe *et al.* 1987). It is a feature of the so-called 'chronic rejection' in renal allografts (Fellstrom *et al.* 1993) where gradual progressive occlusion of the renal vasculature eventually leads to failure of the grafted organ.

2 b) CELLS INVOLVED IN INTIMAL HYPERPLASIA

As with atherosclerosis, the key cell in intimal hyperplasia is the vascular smooth muscle cell. The pathological lesion consists of an accumulation of smooth muscle cells within an extracellular matrix. Smooth muscle cells can exist in either a contractile or a secretory phenotype (Campbell *et al.* 1981). Intimal smooth muscle cells are typically of a secretory phenotype, as opposed to the contractile phenotype found in the media of normal vessels (Painter 1991a). In the contractile state, the smooth muscle cell can contract when stimulated to do so by an agent such as angiotensin. However, after a phenotypic change the smooth muscle cell loses its thick myosin filaments and develops a Golgi apparatus more suited to a secretory function (Chamley-Campbell *et al.* 1981b). This change is initially reversible (Campbell *et al.* 1981).

Figure 2a.1 Transverse section of vein graft stenosis x40 (Smooth muscle actin and Miller's elastin).



The smooth muscle cell can only proliferate when in its secretory phenotype (Chamley-Campbell *et al.* 1981b). Thus SMC proliferation is largely confined to the intima where most of the cells are in a secretory state. The well developed Golgi apparatus of these cells is necessary for the production of proteins needed for cell proliferation and also for the production of autocrine growth factors. Intimal arterial SMCs in the secretory phenotype secrete more PDGF when placed in culture than unmanipulated SMCs from the media of the same arteries (Walker *et al.* 1986). In rats, mRNA for the PDGF- receptor is expressed only in secretory SMCs (Sjolund *et al.* 1990), as is non-muscle myosin which can be used as a marker for restenosis after angioplasty (Leclerc *et al.* 1992). The extracellular matrix of glycosaminoglycans, collagen and elastin typical of intimal hyperplasia is produced by these secretory SMCs (Painter 1991a).

In addition to proliferation, there may be some migration of smooth muscle cells. The intima of arteries does not normally contain SMCs, although macroscopically-normal veins may have smooth muscle cells in their intima on histological examination (Marin *et al.* 1994, Varty *et al.* 1995). This anatomical difference suggests that the mechanisms involved in vein

graft intimal hyperplasia may not be the same as those in arterial intimal hyperplasia. In order to move from the media to the intima, SMCs have to degrade the extracellular matrix in which they lie. Smooth muscle cells are able to produce a number of proteolytic enzymes which allow them to do this. The principal enzymes produced by SMCs to degrade the matrix are matrix metalloproteinases (MMPs). This group of enzymes consists of nine or more endopeptidases which share a number of structural features (Birkedal-Hansen *et al.* 1993) (Table 2b.1). All of these enzymes have a zinc-binding site in the catalytic domain. Vascular SMCs in culture will express mRNA for collagenase and stromelysin if mechanically injured (James *et al.* 1993). Zymography has also shown increased production of 72kd and 92kd gelatinases in rat arterial SMCs after balloon angioplasty (Zempo *et al.* 1994). Heparin, which has long been known to inhibit various models of intimal hyperplasia (Clowes *et al.* 1977), inhibits the induction of stromelysin, 92kd gelatinase and collagenase by primate arterial SMCs (Kenagy *et al.* 1994). This may be one way in which heparin acts to reduce intimal hyperplasia. Inhibitors of MMPs have been shown to reduce intimal hyperplasia in the balloon-injured rat carotid artery model (Bendeck *et al.* 1994) and in explants of rabbit aorta (Southgate *et al.* 1992).

The vascular endothelium is in intimate contact with the blood and is separated from the smooth muscle cells of the media by a thin basement membrane. Under normal physiological conditions it acts as a barrier against the various mitogens present in serum and, as it is non-thrombogenic, it prevents the adhesion of platelets to the vessel wall (Davies *et al.* 1993b). Intimal hyperplasia usually follows an injury to the endothelium. The degree of SMC proliferation is more closely related to the degree of injury than the extent of endothelial denudation (Painter 1991b). Indeed the endothelium does not need to be denuded for intimal hyperplasia to occur and can be morphologically intact (Williams 1991, Reidy *et al.* 1992b). In organ culture models of intimal hyperplasia of both arteries (Koo *et al.* 1991) and veins (Angelini *et al.* 1991), intimal proliferation does not occur if the endothelium is removed. Work in our Department has suggested that a paracrine factor may be produced by the endothelium which is necessary for this intimal growth (Allen *et al.* 1994). This factor is able to stimulate intimal proliferation in cultured vein which has been denuded of endothelium.

Table 2b.1 Matrix Metalloproteinases found in human tissue (Birkedal-Hansen *et al.* 1993)

Metalloproteinase	Alternative name	Substrates
MMP1	Fibroblast-type collagenase	Collagen (types I-III, VII, VIII, X), Gelatin, Proteoglycan core protein.
MMP2	72K Gelatinase Type IV Collagenase	Gelatin, Collagen (types IV, V, VII, X, XI), Elastin, Fibronectin, Proteoglycan core protein
MMP3	Stromelysin-1	Proteoglycan core protein, Fibronectin, Laminin, Collagen (types IV, V, IX, X), Elastin,
MMP7	Putative Metalloproteinase-1	Fibronectin, Laminin, Collagen (type IV), Gelatin, Proteoglycan core protein
MMP8	*PMN-type Collagenase	as MMP1
MMP9	92K Gelatinase Type IV Collagenase	Gelatin, Collagen (Types IV, V), Elastin, Proteoglycan core protein
MMP10	Stromelysin-2	as MMP3
MMP11	Stromelysin-3	not known
	Macrophage metalloelastase	Elastin

*PMN = Polymorphonuclear cell

Whilst endothelium is capable of promoting intimal hyperplasia by the production of the vasoconstrictor endothelin-1 (ET-1), as well as various growth factors, it also has a growth inhibitory rôle by preventing macrophages and platelets from adhering to the blood vessel (Thompson *et al.* 1994a) and by producing growth inhibitory substances such as prostacyclin and Endothelial-Derived Relaxing Factor (EDRF) (Newby *et al.* 1992, Komori *et al.* 1991, Lerman *et al.* 1992). The overall effect of the endothelium on intimal hyperplasia results from a balance between these factors. The restoration of an intact endothelial layer onto a balloon-angioplastied carotid artery in a rabbit model is able to reduce the formation of intimal hyperplasia (Thompson *et al.* 1994b). In an *in vitro* model of endothelial cell seeding, the seeded cells have been shown to produce prostacyclin (Thompson *et al.* 1992). The production of prostacyclin in animal models of vein grafts is often reduced (Komori *et al.* 1990), possibly because of trauma to the endothelium in harvesting the vein. Human saphenous vein harvested for bypass surgery similarly shows reduced production of EDRF and prostacyclin (Angelini *et al.* 1987, Sayers *et al.* 1992b). This damage can be minimised by careful dissection with minimal handling of the vein (Angelini *et al.* 1992a) and by using a preservation medium containing either papaverine or heparinized blood (LoGerfo *et al.* 1981a, Lawrie *et al.* 1990, Dion *et al.* 1990).

Platelets are predominantly concerned with haemostasis. They will adhere to injured endothelium in a cut blood vessel and form a 'plug' to reduce bleeding. They also produce growth factors to stimulate repair of the damaged blood vessel. This is a normal physiological process but platelets act in a similar manner in the pathological process of intimal hyperplasia (Ip *et al.* 1991). Platelets are able to produce Platelet-Derived Growth Factor (PDGF), Epithelial Growth Factor (EGF), Insulin-like Growth Factor-1 (IGF-1), and Transforming Growth Factor beta (TGF-β), all of which are capable of stimulating SMC proliferation (Hwang *et al.* 1992). However, the importance of platelets in promoting intimal hyperplasia *in vivo* is of some doubt. In the rat model of balloon-injured carotid artery, thrombocytopaenia leads to decreased platelet deposition on the denuded carotid artery after angioplasty. There is decreased intimal hyperplasia in the first 7 days but no decrease in SMC proliferation (Fingerle *et al.* 1989) suggesting that platelets may be important for initial SMC migration into the intima but are not required for the subsequent replication of the SMCs.

2 c) GROWTH FACTORS INVOLVED IN INTIMAL HYPERPLASIA

A growth factor stimulates or promotes growth of a cell or group of cells. A cytokine promotes movement of a cell or group of cells, particularly inflammatory cells. The terms are often used interchangeably in vascular biology as some previously known cytokines have been shown to cause proliferation of vascular cells. Several growth factors have been shown to cause proliferation of vascular smooth muscle cells in cell culture, tissue culture and animal models. They have been implicated in intimal hyperplasia and atherosclerosis. Growth factors are produced by platelets, macrophages and the vascular smooth muscle cells and endothelium themselves. The cells that produce the relevant growth factors are shown in Table 2c.1.

2 c.i) PLATELET-DERIVED GROWTH FACTOR

Platelet-derived growth factor (PDGF) is probably the best characterised growth factor. It is a glycoprotein with a molecular weight of between 27-31kDaltons (kD). It was first recognised by Ross et al. in the early 1970's (Ross *et al.* 1974) and was found to be mitogenic for arterial smooth muscle cells.

PDGF is a dimeric compound consisting of 2 covalently linked subunits which are either 'A' (16kD) or 'B' (14kD) chains. The 2 chains are 60% homologous in sequence and are encoded by separate genes (Westermark *et al.* 1989). The A chain is encoded on the long arm of chromosome 7 and the B chain on chromosome 22 (Ross 1989). The B chain is encoded by the c-sis proto-oncogene, the homologue of the oncogene encoded by the simian sarcoma virus (Waterfield *et al.* 1983). Indeed, antibodies directed against PDGF have been able to prevent cell transformation by the simian sarcoma virus (Johnsson *et al.* 1985). Both A and B chains have been shown to have some mitogenic activity (Hosang *et al.* 1989b), though they do have some specific biological differences (Sachinidis *et al.* 1990). The dominant form of PDGF produced by human platelets is PDGF-AB. This comprises about 70% of the platelets' production, with the

Table 2c.1 Growth Factors and the cells known to produce them in humans

Growth Factor	Cell type known to produce factor
Platelet-Derived Growth Factor	Platelets, Endothelial Cells, SMCs
Basic Fibroblast Growth Factor	Endothelial Cells, SMCs, Macrophages
Transforming Growth Factor beta	Platelets, Macrophages, Endothelial Cells, SMCs
Tumour Necrosis Factor alpha	Macrophages, SMCs
Interleukin-1 alpha	Macrophages, SMCs, Endothelial Cells
Insulin-like Growth Factor-1	Endothelial Cells, SMCs, Platelets, Macrophages
Epidermal Growth Factor	Platelets
Endothelin	Endothelial Cells, SMCs

remainder being mainly PDGF-BB with a small amount of PDGF-AA (Ross 1989). In contrast, porcine platelets produce predominantly PDGF-BB (Hart *et al.* 1988). PDGF is not only produced by platelets but also by regenerating endothelial cells, (Limanni *et al.* 1988, Fox *et al.* 1991, Liaw *et al.* 1993) activated macrophages (Besner *et al.* 1990) and SMCs (Walker *et al.* 1986, Golden *et al.* 1991, Hahn *et al.* 1991). Endothelial cells produce predominantly PDGF-BB (Kourembanas *et al.* 1989), whilst SMCs produce predominantly PDGF-AA (Winkles *et al.* 1991).

There are 2 types of PDGF receptor, termed an A/B receptor and a B receptor. The A/B receptor binds the AA, AB and BB dimers whilst the B receptor binds only the BB dimer (Hart *et al.* 1988). Thus PDGF-AA is only able to stimulate the A/B receptor whilst PDGF-BB is able to stimulate both the A/B and the B receptor. PDGF receptors are found in a wide range of cells. Smooth muscle cells from intimal hyperplastic lesions in human grafts have been shown to possess PDGF receptors as well as producing PDGF-AA (Birinyi *et al.* 1989) themselves.

2 c.ii) BASIC FIBROBLAST GROWTH FACTOR

Basic fibroblast growth factor (bFGF) is mitogenic for a wide range of mesenchymal cells. It is a 16kD protein sharing a 55% homology in amino acid sequence with acidic fibroblast growth factor (Gospodarowicz 1990).

A number of cells are capable of producing bFGF. These include vascular endothelial cells (Hannan *et al.* 1988, Lindner *et al.* 1989), vascular smooth muscle cells (Cordon Cardo *et al.* 1990) and macrophages (Greisler *et al.* 1993). There is no signal sequence for secretion of basic FGF (D'Amore 1990b). It is a cell associated protein which is deposited in the basement membrane of the cell (Folkman *et al.* 1988). It is present in the basement membrane of small, medium and large size vessels in humans (Cordon Cardo *et al.* 1990). It may be released by cellular injury or by the action of heparan sulphate-degrading enzymes or heparin produced during inflammatory processes (D'Amore 1990a). It has a high affinity for heparin and heparin-related compounds. It was formerly referred to as Heparin-Binding Growth Factor-2 (Acidic FGF was Heparin-Binding Growth Factor-1).

Basic FGF has a proliferative effect on both endothelial and smooth muscle cells. It also promotes cell migration in SMCs (Jackson *et al.* 1993). Removal of bFGF from the culture medium leads to apoptosis of cultured Human Umbilical Vein Endothelial Cells (HUVECs) (Araki *et al.* 1990), suggesting that it is necessary to keep the cells alive. Basic FGF expression is increased in regenerating endothelial cells (Lindner *et al.* 1989) and antisense to bFGF will suppress proliferation of Bovine Aortic Endothelial Cells (BAECs) in culture (Itoh *et al.* 1992). Growth factors do not work alone and bFGF has important synergy with other growth factors. Interleukin-1 (IL-1) and Tumour Necrosis Nactor alpha (TNF-α) exert an antiproliferative effect on endothelial cells but stimulate SMC proliferation by respectively downregulating and upregulating their bFGF receptors (Sawada *et al.* 1990). Interleukin-1 also stimulates bFGF gene expression in human saphenous vein SMCs (Gay *et al.* 1991). Basic FGF upregulates endothelin-1 receptors in vascular smooth muscle cells (Cristiani *et al.* 1994) which, in the presence of endothelin will promote proliferation. PDGF-B chain production by HUVECs is

reduced by bFGF (Kourembanas *et al.* 1989) whilst PDGF will stimulate bFGF mRNA production in bovine aortic smooth muscle cells (Sato *et al.* 1991). In addition the ability of PDGF to promote migration of the SMCs is abolished by an antibody to bFGF, implying that migration of SMCs may be stimulated by endogenous bFGF induced by PDGF (Sato *et al.* 1991). Basic FGF will also induce mRNA for the alpha receptor for PDGF in vascular smooth muscle cells (Schollmann *et al.* 1992). PDGF-AA is not as potent a mitogen as PDGF-AB or PDGF-BB, but when the number of receptors it can act on are increased, its overall potency is enhanced. Angiotensin II exerts some of its proliferative effect on SMCs by its ability to induce bFGF synthesis (Itoh *et al.* 1993) and enhance the mitogenicity of both PDGF and bFGF (Ko *et al.* 1993).

Cuevas et al. showed that infusion of bFGF directly into the media of a normal rat carotid artery will produce SMC proliferation and neovascularisation *in vivo* (Cuevas *et al.* 1991). However, other workers have shown no effect of bFGF on normal, unmanipulated rat carotid artery but a significant proliferative response following balloon denudation of the artery (Lindner *et al.* 1991a, Edelman *et al.* 1992). Indeed, Lindner et al. showed an increase in proliferation from 11.5% to 54.8% after balloon injury and from 1.3% to 43.3% following a milder form of endothelial injury using a nylon loop (Lindner *et al.* 1991a). Systemic injection of a neutralising antibody to bFGF before balloon angioplasty of the rat carotid artery reduces the SMC proliferation by about 80% but has no effect on the degree of intimal hyperplasia seen at 8 days (Lindner *et al.* 1991b). If the neutralising antibody is given at 4 or 5 days after angioplasty, there is no effect on SMC proliferation (Olson *et al.* 1992), and antibodies to bFGF or to PDGF have no effect on the proliferation of intimal SMCs (Reidy 1992a). The migration of SMCs from the balloon-injured media of the rat carotid artery into the intima is influenced by bFGF. Systemic bFGF increases the rate of migration whilst blocking antibodies to bFGF reduce the rate of migration (Jackson *et al.* 1993).

In the organ culture model of intimal hyperplasia in human saphenous vein described by Soyombo et al., the proliferative response of the SMCs to surgical preparation of the vein is reduced by a neutralising antibody to bFGF (Soyombo *et al.* 1993b).

2 c.iii) TRANSFORMING GROWTH FACTOR BETA

Transforming Growth Factor Beta (TGF-β) is a 25kD homodimeric peptide with a range of functions affecting cell proliferation and differentiation in a number of different cell types (Sporn *et al.* 1987, Border *et al.* 1995). There are 2 distinct forms, termed TGF-β1 and TGF-β2. The type found predominantly in human tissues is TGF-β1. A wide range of cells produce and bear receptors for transforming growth factors, including platelets (Miyazono *et al.* 1989), macrophages, endothelial (Hannan *et al.* 1988) and smooth muscle cells. The neointimal SMCs in the rat carotid artery express increased levels of mRNA for TGF-β within 6 hours of balloon angioplasty (Majesky *et al.* 1991). TGF-β can have both proliferative and antiproliferative effects on vascular tissues.

The effect of TGF-β on SMCs is bimodal. At low doses, on subconfluent cells, TGF-β has a stimulatory effect on SMC growth. At higher concentrations, the effect is inhibitory. There is a similar bimodal effect on SMC migration with enhancement of migration at low concentrations of 10-50pg/ml and inhibition at higher concentrations of 1pg/ml-1ng/ml (Koyama *et al.* 1990). At low concentrations, TGF-β induces production of PDGF-AA (Majack *et al.* 1990, Winkles *et al.* 1991), hence a latent period of around 24 hours before proliferation occurs. At higher concentrations, TGF-β decreases transcripts and expression of PDGF receptor alpha subunits (Battegay *et al.* 1990), thereby inhibiting proliferation.

Endothelin mRNA levels and endothelin-1 secretion into solution are increased in cultured porcine aortic endothelial cells by TGF-β (Kurihara *et al.* 1989).

Transforming Growth Factor beta also plays a role in producing extracellular matrix. It stimulates the production of types I and III collagen by vascular smooth muscle cells (Amento *et al.* 1991, Schlumberger *et al.* 1991). SMCs exposed to TGF-β in culture also show a greater degree of myodifferentiation with increased expression of filamentous alpha-smooth muscle actin (Bjorkerud 1991). One report in the literature even suggested that endothelial cells could be stimulated to express alpha-smooth muscle actin by the action of TGF-β (Arciniegas *et al.* 1992).

2 c.iv) TUMOUR NECROSIS FACTOR ALPHA

Tumour Necrosis Factor alpha (TNF-α) is a 17kD protein that exists as a multimer of 2, 3 or 5 non-covalently linked units. It is secreted by lipopolysaccharide-stimulated macrophages and derives its name from its ability to promote tumour necrosis in tumour-bearing mice *in vivo* (Carswell *et al.* 1975). Human vascular SMCs derived from the intima of atherosclerotic plaques express mRNA for TNF-α and stain for the TNF-α protein using immunohistochemical techniques (Barath *et al.* 1990). Whilst normal medial SMCs do not express mRNA for TNF-α, incubation of SMCs with inhibitors of protein synthesis and lipopolysaccharide leads to both mRNA expression and production of the protein product of TNF-α (Warner *et al.* 1989).

There are several mechanisms by which TNF-α may promote intimal hyperplasia. In conjunction with Interleukin-1 (IL-1), TNF-α upregulates bFGF receptors on SMCs and downregulates bFGF receptors on endothelial cells (Sawada *et al.* 1990). The cytokine IL-1 is able to stimulate the autocrine production of PDGF-AA by vascular SMCs. The production of IL-1 is increased in both vascular endothelial cells (Shingu *et al.* 1991) and vascular SMCs (Warner *et al.* 1989, Shingu *et al.* 1991) by the action of recombinant TNF-α. This IL-1 tends to remain cell associated on the cell surface where it is able to exert a paracrine effect (Loppnow *et al.* 1992). The production of c-sis protooncogene mRNA (the PDGF-B chain) in human vascular endothelial cells is also enhanced by TNF-α (Calderon *et al.* 1992).

Macrophages and monocytes release a number of products which promote intimal hyperplasia (Greisler 1989). TNF-α increases mRNA expression of ICAM-1 and cell surface ICAM-1 on human aortic SMCs (Couffinhal *et al.* 1993). This allows an increased adherence of monocytes to the SMCs (by a factor of 2 in this experiment by Couffinal et al.). There is also an increase in VCAM-1 mRNA and cell surface expression of VCAM-1 in SMCs treated with TNF-α (Couffinhal *et al.* 1994).

Vascular SMCs from human veins increase mRNA expression of the matrix metalloproteinases MMP-3 (Stromelysin) and MMP-9 (Gelatinase B) in response to TNF-α (Hanemaaijer *et al.* 1993). These MMPs allow degradation of the extracellular matrix necessary for migration of the SMCs.

2 c.v) INTERLEUKIN-1

Interleukin-1 (IL-1), originally known as lymphocyte-activating factor, plays a key role in inflammatory and immune responses. It activates T-cells and lymphocytes, causing them to proliferate and produce Interleukin-2 (IL-2). It is released by stimulated macrophages and monocytes but can also be produced by vascular SMCs (Libby *et al.* 1986b) and endothelial cells (Libby *et al.* 1986a). It is not expressed in healthy human veins or in normal internal mammary artery, but is expressed in phlebosclerotic veins and in the veins of occluded coronary artery bypass grafts (Brody *et al.* 1992). There are 2 closely related compounds, IL-1 α and IL-1β which elicit nearly identical biological responses and are 62% homologous in their amino acid sequences. They both have a molecular weight of 17kD.

Whilst IL-1 was known to stimulate proliferation of fibroblasts, it was initially thought to have no effect on the proliferation of SMCs (Libby *et al.* 1985b). This is because IL-1 also stimulates SMCs to produce the growth-inhibiting prostanoids Prostaglandin E1 (PGE1) and Prostaglandin E2 (PGE2). However, Libby et al. later showed that if SMCs were cultured with IL-1 in the presence of a cyclo-oxygenase inhibitor or were cultured for 7-28 days rather than a mere 2 days, then IL-1 could stimulate proliferation of SMCs (Libby *et al.* 1988a). Interleukin-1 has several modes of action which may promote intimal hyperplasia. There is a direct effect on SMC proliferation. This action is delayed by some 8 hours relative to the proliferative response elicited by PDGF, and it is inhibited by a blocking antibody to PDGF (Raines *et al.* 1989, Ikeda *et al.* 1990). This proliferation is also accompanied by the expression of mRNA for the PDGF-A chain in the SMCs (Raines *et al.* 1989), strong evidence that the proliferative action of IL-1 is largely due to endogenous PDGF production by the SMCs.

Interleukin-1 is also able to stimulate endothelial cells and SMCs to produce Interleukin-6 (Loppnow *et al.* 1989, Norioka *et al.* 1990), which can independently cause an increase in c-myc mRNA expression, increased DNA synthesis and an increase in cell numbers of SMCs (Morimoto *et al.* 1991). Interleukin-6 also promotes vascular SMC proliferation by stimulating endogenous production of PDGF (Ikeda *et al.* 1991) and by inducing expression of c-sis protooncogene mRNA in human vascular endothelial cells (Calderon *et al.* 1992).

The production of the matrix metalloproteinase MMP1 (Tissue collagenase) by human aortic SMCs is increased by both PDGF and IL-1 (Saltis *et al.* 1992). As collagenase helps to break down extracellular matrix, this production can help cells to migrate out of the media and into the intima of the vessel.

2 c.vi) INSULIN-LIKE GROWTH FACTOR-1

Insulin-like Growth Factor-1 (IGF-1) is a 7.7kD protein which has a number of growth-regulatory properties in different cell types. It can be produced by endothelial cells, vascular smooth muscle cells (Delafontaine *et al.* 1991a), platelets and macrophages (Ferns *et al.* 1991). It is able to increase tritiated thymidine uptake by a factor of 6 in pulmonary artery SMCs (Dempsey *et al.* 1990). Its mitogenic action on vascular SMCs is synergistic with that of PDGF (Banskota *et al.* 1989, Ko *et al.* 1993, Yamamoto *et al.* 1994). IGF-1 also has a chemotactic effect on vascular SMCs which is distinct from its mitogenic effect and is blocked by a blocking antibody to the IGF-1 receptor (Bornfeldt *et al.* 1994). The secretion of IGF-1 by endothelial cells is polarised towards the basal rather than the apical surface (Taylor *et al.* 1993). This basal secretion is directed towards the SMCs in the media of the blood vessel where it is able to exert a paracrine effect.

PDGF has been shown to have varying effects on the expression of mRNA for IGF-1 in vascular SMCs. Delafontaine et al. showed an increase in IGF-1 mRNA expression when rat aortic SMCs were stimulated with either PDGF or serum (Delafontaine *et al.* 1991b). Gianella-Neto et al. showed a decrease in IGF-1 mRNA expression with PDGF or with increasing cell confluence in rat aortic SMCs (Giannella-Neto *et al.* 1992), with an associated increase in the

production of an inhibitory IGF-binding protein. Other authors have shown an increase in the levels of mRNA for the IGF-1 receptor in response to stimulation with PDGF (Ververis *et al.* 1993, Rubini *et al.* 1994).

Bornfeldt et al. showed increased expression of mRNA for both IGF-1 and its receptor in balloon-injured rat aorta (Bornfeldt *et al.* 1992), whilst Khorsandi et al. showed increased expression of IGF-1 mRNA but a decrease in mRNA for the IGF-1 receptor in the same model. In-situ hybridisation showed this change in mRNA expression to be predominantly in the medial SMCs (Khorsandi *et al.* 1992).

2 c.vii) ENDOTHELIN

Endothelin-1 (ET-1) is a 21 amino acid peptide. It is the predominant form of endothelin found in humans. It was first recognised as a potent endothelial-derived vasoconstrictor in the late 1980s (Yanagisawa *et al.* 1988).

In addition to its vasoconstricting ability, ET-1 was shown to stimulate expression of mRNA for the protooncogenes c-fos and c-myc in vascular SMCs (Komuro *et al.* 1988) and to be mitogenic for rat vascular smooth muscle cells (Hirata *et al.* 1989). The ET-1 induced synthesis of DNA by vascular SMCs is enhanced by the presence of PDGF and inhibited by the calcium antagonist nifedipine (Nakaki *et al.* 1989). Weissberg et al. showed that the closely related compounds ET-1, ET-2, ET-3 and sarafotoxin were all comitogenic with PDGF for rat vascular SMCs but were unable to stimulate growth of the rat vascular SMCs in the absence of PDGF (Weissberg *et al.* 1990).

Work in our Department by Masood et al. (unpublished) has shown that recombinant endothelin-1, when added to culture medium containing 30% foetal calf serum, is able to stimulate intimal SMC proliferation in segments of cultured human saphenous vein which have been denuded of their endothelium.

2 c.viii) EPIDERMAL GROWTH FACTOR

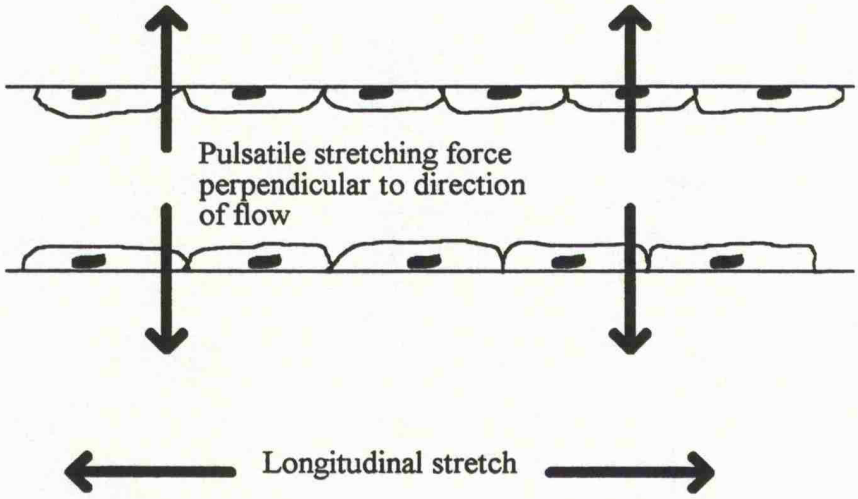
Epidermal growth factor (EGF) is a 6kD peptide growth factor closely related to transforming growth factor alpha. It is produced and released by activated platelets. A heparin-binding EGF-like compound (HB-EGF) is also produced by macrophages and SMCs in human atherosclerotic plaques (Miyagawa *et al.* 1995) as well as cultured human vascular SMCs (Higashiyama *et al.* 1994).

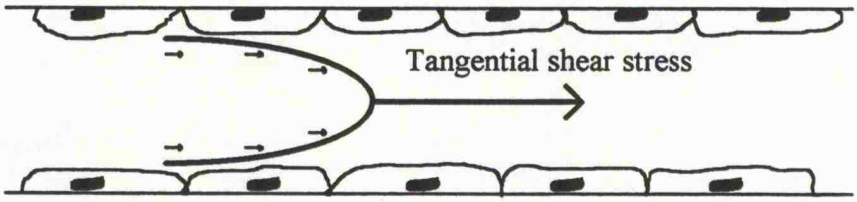
Receptors for EGF have been found on rat aortic SMCs and EGF has been shown to cause proliferation of these cells in culture (Tomita *et al.* 1986) which is inhibited by the action of calcium antagonists (Tomita *et al.* 1987). However, whilst EGF will cause rapid DNA synthesis in cultured subconfluent SMCs in 0.4% serum, there is relatively little increase in cell numbers (Bagby *et al.* 1992). EGF is not a complete mitogen and needs various serum factors to complete cell division and proliferation. Human foreskin fibroblasts in culture will increase expression of mRNA to c-fos and c-myc in response to stimulation with either EGF or PDGF (Paulsson *et al.* 1987). In cultured porcine aortic SMCs, EGF also has an additive effect on the production of collagen stimulated by TGF-β (Schlumberger *et al.* 1991).

2 d) THE EFFECT OF FLOW ON INTIMAL HYPERPLASIA

Cell and organ culture models of vascular tissue are often criticised because of their static nature. Under *in vivo* conditions, blood vessels are subjected to cyclic stretch and to shear stress from the movement of fluid in the vessel. Arteries, because of their high rate of flow and high pulsatile pressure, are subjected to higher shear stress, greater changes in shear stress and greater changes in cyclic stretch than veins, where the flow rate is less and cyclic stretch is minimal. There are difficulties in examining the effects of pulsatile pressure and flow *in vivo* in humans but there are several methods available to study the effects of shear stress and cyclic stretch on isolated vascular tissues and in animal models.

Figure 2d.1 Diagrammatic representation of the 2 different actions of flow: Cyclic stretch and Shear stress.





2 d.i) THE EFFECT OF CYCLIC STRAIN ON INTIMAL HYPERPLASIA

Isolated cells can be subjected to cyclic stretch on a deformable plate in a culture system. This can provide information on cell signals initiated by stretch alone without the influence of flow-induced shear stress. There is a time-dependent increase in adenylate cyclase production in bovine aortic endothelial cells (BAECs) stimulated by cyclical strain (Letsou *et al.* 1990) whilst similar stretching of porcine coronary artery SMCs leads to a 30% reduction in adenylate cyclase production (Mills *et al.* 1990). Human saphenous vein endothelial cells on the other hand show no changes in cyclic AMP in response to stretch (Iba *et al.* 1992a). Cyclic stretch may exert its effects by its action on protein kinase C. There is a translocation of protein kinase C from the cytosol to the perinuclear and nuclear regions of the bovine aortic

endothelial cell. In addition, calphostin C (a specific protein kinase C inhibitor) abolishes the proliferative effect of cyclic stretch on endothelial cells (Rosales *et al.* 1992a). Phospholipase C activation is another potential method of mechanotransduction. Inositol triphosphate and diacylglycerol, metabolites of Phospholipase C, are both transiently increased by an initiation of or an acute increase (Rosales *et al.* 1992b) or decrease (Brophy *et al.* 1993) in frequency of cyclic stretch.

Cyclical strain stimulates endothelial cells to proliferate (Widmann *et al.* 1992) and increase production of endothelin (Sumpio *et al.* 1990b), itself a mitogen for SMCs. Cyclical stretching of cultured BAECs causes an inhibition of collagen production (Sumpio *et al.* 1990a) whilst similar stretching of rabbit aortic SMCs induces production of collagen, hyaluronate and chondroitin-6-sulphate (Leung *et al.* 1976). The reaction of endothelial cells from different regions of the vascular tree to cyclical strain may vary. Human saphenous vein endothelial cells exposed to cyclic stretch align their actin fibres perpendicular to the direction of the force vector. After 12 hours of such stress, the cells themselves become elongated and orientated in the same direction as the actin filaments (Iba *et al.* 1991c). Iba et al. showed that bovine endothelial cells from the vena cava react in the same way, but endothelial cells from the pulmonary artery and aorta of the same animal showed no morphological changes in response to cyclic stretch (Iba *et al.* 1991a), implying that endothelial cells from different parts of the vascular tree respond differently to mechanical stress. This contrasts with the report by Kanda et al. who showed that endothelial cells, SMCs and fibroblasts from the bovine arterial wall all aligned themselves perpendicular to the direction of stretch in culture (Kanda *et al.* 1993), although the endothelial cells responded less rapidly.

Human saphenous vein endothelial cells respond to pulsatile stretch with increased production of tissue plasminogen activator which may be important in maintaining the low thrombogenicity of vein grafts *in vivo* (Iba *et al.* 1991b, Iba *et al.* 1992b). BAECs show increased nitric oxide synthase activity in response to cyclic stretch (Awolesi *et al.* 1994) which also helps to reduce thrombogenicity.

Smooth muscle cells from different parts of the vascular tree also appear to act in different ways in response to cyclic stretch. Predel et al. showed that SMCs derived from human saphenous vein were stimulated to proliferate by pulsatile stretch but SMCs derived from the internal mammary artery of the same patient were not (Predel *et al.* 1992). He concluded that the differential responses of arterial and venous SMCs may partially explain the improved patency of internal mammary as opposed to saphenous vein grafts in coronary artery bypass surgery.

The growth factors PDGF and bFGF are both well known SMC mitogens. Malek et al. reported that cyclical stretch of BAECs had no effect on the expression of mRNA for these factors whilst increasing shear stress decreased the expression of PDGF-B mRNA and increased the expression of bFGF mRNA (Malek *et al.* 1993a). This increase in bFGF was small (x1.5) and transient at a shear stress of 15 dynes/cm^2 but was more significant and more prolonged at a higher shear stress of 36 dynes/cm^2. In contrast, cyclic stretch of neonatal rat SMCs leads to an increase in tritiated thymidine uptake with increased secretion of both PDGF-AA and PDGF-BB (Wilson *et al.* 1993). The proliferation induced by the cyclical stretch in this rat model could be partially blocked by polyclonal antibodies to PDGF-AA (75%) and PDGF-BB (50%).

As further evidence that cyclic stretch is important in intimal hyperplasia, an *in vitro* model of a PTFE to artery anastomosis showed that the area of maximal cyclical strain at the anastomotic region of the artery corresponded to the area most commonly affected by anastomotic intimal hyperplasia (Pevec *et al.* 1993).

An example of cyclic stretch having an effect on SMC growth *in vivo* and not just *in vitro* is the Spontaneously Hypertensive Rat, where a reduction in pulse pressure leads to a decrease in medial SMC growth (Christenson 1991).

2 d.ii) THE EFFECT OF SHEAR STRESS ON INTIMAL HYPERPLASIA

Whilst the overall effect of pulsatile or cyclical stretch on vascular tissue is to promote intimal hyperplasia, various models of shear stress suggest that the effect of flow *per se* is to reduce intimal hyperplasia. Shear stress (τ) is the tractive force exerted on the vessel wall by the flow through the vessel. It is proportional to the velocity of flow (Q), the viscosity of the medium (η), and inversely proportional to the cube of the radius of the vessel (r) as derived from the Hagen-Poiseuille formula:

$$\tau = 4\eta Q / \pi r^3$$

This formula assumes laminar flow, which is rarely the case in human blood vessels, but it does provide a close approximation. The tractive force is exerted primarily on the endothelium and any effects on the blood vessel are almost certainly mediated through the endothelium.

There are several possible mechanisms by which the endothelial cells may mediate the response to shear stress. There is a rapid potassium-selective shear stress-activated current in cultured endothelial cells which causes hyperpolarisation in the presence of high shear (Olesen *et al.* 1988). There is also a rise in intracellular calcium (Nerem *et al.* 1990) which occurs rapidly after application of shear stress. This rise is not affected by extracellular calcium concentrations or by calcium antagonists (Ando *et al.* 1990b, Geiger *et al.* 1992). This rise in intracellular calcium only occurs in the presence of ATP, ADP or AMP (Ando *et al.* 1991, Dull *et al.* 1991, Mo *et al.* 1991, Ando *et al.* 1993b). Inositol triphosphate production, another possible cellular signal mechanism, is also increased by shear stress (Nollert *et al.* 1990, Bhagyalakshmi *et al.* 1992), as it is by cyclic stretch.

In the 1960's, Fox & Hugh proposed a theory of atheroma developing at areas of low shear stress (Fox *et al.* 1966). In the 1970's Faulkner et al. demonstrated in a canine model that intimal hyperplasia in autologous vein grafts was more pronounced when the flow was decreased and was reduced by an increase in flow (Faulkner *et al.* 1975). Rittgers et al. came to similar conclusions also using a canine model (Rittgers *et al.* 1978). Restoration of normal flow after development of intimal hyperplasia can lead to regression of the neointima in this model

(Morinaga *et al.* 1987). The intimal hyperplastic response following balloon denudation or laser thermal damage of the arterial endothelium is also exacerbated by poor vascular run-off (Hehrlein *et al.* 1991), where flow and shear stress are reduced.

The Seattle group led by Dr A. Clowes have performed numerous experiments using a model of intimal hyperplasia occurring in a PTFE graft in a baboon. Intimal hyperplasia occurs at approximately 1 month following surgery or an acute reduction in graft flow (Geary *et al.* 1994a). The degree of intimal hyperplasia is increased if the flow and therefore shear stress is lowered. Conversely, increased shear produces less intimal growth (Kohler *et al.* 1991, Kraiss *et al.* 1991). Reduced shear is associated with increased expression of mRNA for the PDGF-A chain within the intimal SMCs (Geary *et al.* 1994b).

Intimal hyperplasia in a PTFE graft in a rabbit model is greater when the graft is used as a venous substitute than when used as an arterial substitute (Watase *et al.* 1992) i.e. it is greatest when the shear stress is least.

Low shear stress will stimulate porcine endothelial cells to produce the mitogen endothelin (Yoshizumi *et al.* 1989). Kuchan et al showed that HUVECs exposed to continuous low shear stress < 1.8 dynes/cm^2 or to a sudden increase in shear up to 10 dynes/cm^2 exhibited an increase in endothelin-1 (ET-1) production (Kuchan *et al.* 1993). Continuous exposure to a higher shear stress of between 6-25 dynes/cm^2 led to *decreased* production of ET-1. This effect appears to be mediated by protein kinase C as it is blocked by staurosporine, a protein kinase C antagonist. The expression of preproendothelin (a precursor of endothelin) mRNA in cultured HUVECs and the release of endothelin-1 into solution is reduced by the application of flow (Shareflkin *et al.* 1991). Malek et al. also found that endothelin mRNA expression was downregulated in BAECs by shear stress in a way that was independent of both protein kinase C and cyclic AMP (Malek *et al.* 1993b).

Both constant and pulsatile shear cause a reduction in proliferation of BAECs (Levesque *et al.* 1990, Nerem *et al.* 1990) with a virtual cessation of proliferation at levels of shear stress normally found in arterial flow. If shear stress follows partial denudation of the

endothelium, there is increased DNA production suggesting an increase in cell proliferation (Ando *et al.* 1990a). Conditioned medium from BAECs exposed to shear stress will stimulate SMC migration but has no more a proliferative effect on SMCs than that from conditioned medium from BAECs in static culture (Takakuwa 1990). Shear stress also causes increased production of EDRF from BAECs (Buga *et al.* 1991). Expression of PDGF-A and PDGF-B mRNA is initially increased by increasing shear stress, reaching a peak at 2 hours and falling back to normal levels at 6 hours after initiation of the stress (Hsieh *et al.* 1991). This PDGF gene expression appears to be at least partly dependent upon the action of protein kinase C (Hsieh *et al.* 1992) although Mitsumata et al. showed that expression of PDGF-B mRNA can occur following shear stress even after blocking the action of protein kinase C (Mitsumata *et al.* 1993). Expression of the proto oncogene c-fos is also transiently increased by an increase in shear stress in cultured HUVECs (Hsieh *et al.* 1993). There is also a slight increase in c-myc and c-jun mRNA levels. Although the rise in c-fos level mirrors the rise in PDGF expression, antisense oligonucleotides to c-fos have no effect on PDGF expression.

Whilst in normal vessels, the main cell type exposed to shear stress will be the endothelial cell, SMCs also show various responses to flow. Isolated bovine aortic SMCs exposed to laminar flow show decreased proliferation compared to cells in static culture (Sterpetti *et al.* 1992a, Sterpetti *et al.* 1992c). However, other work by Sterpetti showed that SMCs exposed to shear stress actually released more mitogens (PDGF and bFGF) than cells in static culture (Sterpetti *et al.* 1992b, Sterpetti *et al.* 1993a, Sterpetti *et al.* 1994). He also showed that isolated endothelial cells increased their production of the mitogenic interleukins IL-1 and IL-6 in response to shear stress (Sterpetti *et al.* 1993b).

Tissue plasminogen activator mRNA levels are increased in HUVECs following shear stress (Diamond *et al.* 1990), as they are following pulsatile stretch in human saphenous vein endothelial cells. This again should help maintain the low thrombogenicity of the vessel.

2 e.i) PHARMACOLOGICAL MANIPULATION OF INTIMAL HYPERPLASIA

Several drugs have been used in an attempt to prevent or halt the progression of intimal hyperplasia. These include antiplatelet drugs, calcium antagonists, lipid-lowering drugs, fish oil, heparin, angiotensin-converting enzyme inhibitors, cyclosporin, prazosin and steroids (Varty *et al.* 1993a).

Heparin was one of the first compounds shown to have an inhibitory effect on intimal hyperplasia (Clowes *et al.* 1977). The ability of heparin to inhibit SMC proliferation seems not to be related to its anticoagulant ability (Dryjski *et al.* 1988), as non-anticoagulant fractions of heparin and non-heparin polysulphated molecules are able to inhibit intimal hyperplasia (Paul *et al.* 1987). Although the precise method of heparin's action is not understood, several facts are known. Heparin is able to inhibit both c-fos and c-myc mRNA expression in rat and calf vascular SMCs (Pukac *et al.* 1990). These two protooncogenes are involved in cell proliferation and early inhibition of their expression may be important in preventing intimal hyperplasia (Ebbecke *et al.* 1992). Heparin is also able to bind various growth factors, particularly bFGF (D'Amore 1990a, Lindner *et al.* 1992) and the PDGF-A chain (Fager *et al.* 1992b) and prevent their action on the cell (Fager *et al.* 1992a). The expression of mRNA for bFGF is also inhibited by heparin (Yamaguchi *et al.* 1993). Heparin has an inhibitory effect on matrix metalloproteinase (MMP) expression (Au *et al.* 1993), and therefore reduces the matrix degradation and SMC migration necessary for intimal hyperplasia (Kenagy *et al.* 1994).

Low molecular weight heparin produces a reduction in the intimal growth seen in an organ culture model of human saphenous vein (Varty *et al.* 1994b). However, the concentration of heparin required in the culture medium to have a significant inhibitory effect on intimal growth, far exceeds the safe serum concentration in humans. Low molecular weight heparin does produce a reduction *in vivo* of the intimal hyperplasia seen in a denuded aorta in the New Zealand White rabbit (Wilson *et al.* 1991, Hanke *et al.* 1992). However, clinical studies of the effect of heparin on restenosis in humans after coronary angioplasty have shown no effect (Ellis *et al.* 1989 ; Marin *et al.* 1993b). Cell culture work by Chan et al. suggests that there is wide variation between individuals regarding the responsiveness of their SMCs to

heparin and that patients whose grafts restenose are relatively insensitive to heparin (Chan *et al.* 1993).

A multicentre randomised trial of low molecular weight heparin versus aspirin and dipyridamole, following peripheral arterial bypass surgery, did show an improvement in graft patency for those patients with critical ischaemia who had once daily heparin for 3 months after surgery (Edmondson *et al.* 1994). Actuarial graft survival was improved beyond the 3 months of heparin treatment implying that the improvement was not due to the antithrombotic effect alone. The authors suggested that a reduction in intimal hyperplasia, not seen with the antiplatelet therapy, may have been responsible for the difference between the 2 treatment regimens. However, no improvement in graft survival was shown for those patients operated on for intermittent claudication in this study. In addition, the improvement in graft survival in the critically ischaemic group could not be shown to be associated with a decrease in the number of graft stenoses. It therefore seems unlikely that the improved patency was due to inhibition of intimal hyperplasia.

Platelets were initially thought to have a significant effect on intimal hyperplasia, as they are commonly found on atherosclerotic plaques and are sources of various growth factors. Antiplatelet drugs such as aspirin and dipyridamole have therefore been used in animal and clinical models as inhibitors of intimal hyperplasia. Several authors have looked at venous interposition grafts in the canine femoral artery and have shown varying results with platelet inhibitors. Brody et al. showed no effect on intimal hyperplasia with dipyridamole (Brody *et al.* 1977). This finding was repeated by DeCampli et al. (DeCampli *et al.* 1988), but other workers have shown a beneficial effect on intimal hyperplasia in a canine vein graft model with dipyridamole alone (Landymore *et al.* 1988) or in combination with aspirin (Metke *et al.* 1979, Dobrin *et al.* 1988). A number of studies have shown no effect on animal vein graft models with aspirin alone (Landymore *et al.* 1991, DeCampli *et al.* 1988, Quinones-Baldrich *et al.* 1988b, Boerboom *et al.* 1990, Landymore *et al.* 1990). However, one report of a jugular vein interposition graft in a canine femoral artery did show a reduction in intimal hyperplasia with aspirin (Hirko *et al.* 1993). Aspirin does reduce lipid uptake into vein grafts in hypercholesterolaemic animals (Boerboom *et al.* 1990, Landymore *et al.* 1990) and also leads to

improved vein graft patency in experimental models (Quinones-Baldrich *et al.* 1988b), presumably because of its ability to prevent graft thromboses. The Physicians' Health Study in the U.S.A. showed that aspirin prevented myocardial infarctions but had no effect on the development of angina (Ridker *et al.* 1991), suggesting that it is unable to prevent the progression of atherosclerosis but is able to prevent acute thrombosis because of its antiplatelet action.

Angiotensin II is a potent mitogen for SMCs (Naftilan 1992), therefore drugs which oppose its action have been considered as potential inhibitors of intimal hyperplasia. Those drugs examined include Angiotensin Converting Enzyme Inhibitors (ACE Inhibitors) which block the conversion of angiotensin I to angiotensin II, and the angiotensin receptor antagonist, losartan. ACE inhibitors have shown an ability to reduce intimal hyperplasia in some animal models (Clowes *et al.* 1991b, O'Donohoe *et al.* 1991, Powell *et al.* 1991) but not in others (Hanson *et al.* 1991, Hirko *et al.* 1993). There is no evidence of ACE inhibition having an inhibitory effect on intimal hyperplasia *in vivo* in man (Heyndrickx 1993) though losartan does inhibit intimal hyperplasia in an organ culture model of human saphenous vein (Varty *et al.* 1994a).

Steroids reduce inflammatory processes and inhibit collagen production and might therefore be expected to have an effect on SMC replication. Corticosteroids have been able to reduce intimal hyperplasia in several animal models (Brody *et al.* 1977, Chervu *et al.* 1989, Colburn *et al.* 1992) but no effect has ever been shown in man. There have been case reports of female reproductive steroids causing accelerated intimal hyperplasia. This has occurred with oral contraceptives (Lamy *et al.* 1988) and also with endogenous steroids during pregnancy (Brick 1988). However, in an animal model of allotransplantation, oestradiol had an inhibitory effect on intimal hyperplasia in arterial allografts in the New Zealand white rabbit (Cheng *et al.* 1991).

Lipid-lowering drugs may be expected to have an effect on intimal hyperplasia as lipoproteins are known to have a proliferative effect on SMCs (Libby *et al.* 1985a). Elevated serum lipids also appear to be a risk factor for restenosis after carotid endarterectomy in

humans (Colyvas *et al.* 1992). The lipid-lowering drug simvastatin has been shown to inhibit migration of cultured porcine arterial SMCs (Hidaka *et al.* 1992) and simvastatin, lovastatin and fluvastatin will reduce neointima formation in a rabbit carotid artery (Soma *et al.* 1993). However, there have been no convincing studies of lipid-lowering drugs inhibiting intimal hyperplasia in humans.

Immunosuppressants such as cyclosporin have produced a reduction in intimal hyperplasia in some animal models (Jonasson *et al.* 1988, Hirko *et al.* 1993). Jonasson et al. showed an inhibition of intimal hyperplasia *in vivo* but no effect on cultured SMCs *in vitro* (Jonasson *et al.* 1988) whilst Ferns et al. showed no effect *in vivo* but decreased proliferation of SMCs *in vitro* (Ferns *et al.* 1990). Cyclosporin has been shown alternately to inhibit the induction of DNA synthesis by PDGF (Thyberg *et al.* 1991) and to enhance the induction of DNA synthesis by PDGF (Locher *et al.* 1991a). It has not been shown to have a role in intimal hyperplasia in humans.

Fish oils, particularly those rich in omega-3 fatty acids, appear to have a beneficial effect in reducing the incidence of atherosclerosis and inhibiting various models of intimal hyperplasia (Fitzgerald *et al.* 1989, Israel *et al.* 1992). In cultured rat SMCs, fish oils diminish the stimulatory effects of the mitogens angiotensin II, lipoprotein and PDGF (Locher *et al.* 1991b). In an *in vivo* experiment involving autologous vein grafts in a canine model, cod liver oil has been shown to have no effect in one study (DeCampli *et al.* 1988) and to inhibit intimal hyperplasia even at low doses in another study (Landymore *et al.* 1989).

The alpha-1 adrenergic antagonist prazosin has shown an ability to reduce intimal hyperplasia in an animal model (O'Malley *et al.* 1989, Norman *et al.* 1992), as has the closely related compound doxazosin (Vashisht *et al.* 1992). This effect may be related to the ability of specific alpha-1 adrenergic agonists, such as phenylephrine, to cause a rapid increase in c-fos expression in cultured rat SMCs (Okazaki *et al.* 1994). This effect is blocked by prazosin.

2 e.ii) MOLECULAR APPROACHES

The advent of molecular biology as a diagnostic and therapeutic science has offered new possibilities for the prevention of intimal hyperplasia. It is now feasible to 'switch off' some of the cellular mechanisms involved in SMC proliferation. This can be done by employing antisense technology. This involves the use of a short length of DNA, usually only 15-20 base pairs in length, which is complimentary to a length of messenger RNA required for one of the cellular signalling mechanisms or one of the growth factors involved in proliferation. This antisense length of DNA, known as an oligonucleotide, is small enough to rapidly pass across the cell membrane where it can block the translation of the mRNA message. The precise mechanism of action is not known but it is thought that an antisense-sense duplex is formed which either prevents reading of the single stranded mRNA message by the ribosomes or leads to degradation of the mRNA. The length of mRNA may contain several thousand nucleotides, so that several hundred potential antisense nucleotides could be directed against the mRNA. There is evidence to suggest that not all of the potential antisense oligonucleotides are able to block the mRNA message, and as little as a 2 or 3 base shift can make the difference between an oligonucleotide that inhibits gene expression and one that does not (Epstein *et al.* 1993).

There have been several reports of animal and tissue culture experiments which have shown some promise with antisense technology. Antisense oligonucleotides to the protooncogenes c-myc and c-myb and to the nuclear protein Proliferating Cell Nuclear Antigen (PCNA) have been used in experimental models. These proteins are switched on during cell proliferation and antisense oligonucleotides can prevent this. Antisense to c-myb severely inhibits the entry of bovine SMCs into the S phase of cell division which normally occurs after the addition of serum (Brown *et al.* 1992). Antisense to c-myb or to non-muscle myosin heavy chain inhibits SMC proliferation *in vitro* (Simons *et al.* 1992b) whilst the local delivery of c-myb antisense to the adventitia of a rat carotid artery suppresses the intimal hyperplasia seen *in vivo* after a balloon angioplasty injury (Simons *et al.* 1992a). Antisense oligonucleotides to c-myc suppress the proliferation of human arterial SMCs in culture (Ebbecke *et al.* 1992, Shi *et al.* 1993) and reduce the proliferation and migration of rat arterial SMCs in culture. Antisense to c-myc has also inhibited the intimal hyperplasia *in vivo* in a balloon-injured rat carotid artery

after application to the arterial adventitia in a pluronic gel (Bennett *et al.* 1994), and in a porcine model of coronary artery balloon injury after being introduced via a transcatheter delivery system (Shi *et al.* 1994). Whilst most of the antisense delivered by the transcatheter system was washed away, enough oligonucleotide remained in the vessel for it to be detected up to 3 days later. This method could therefore be used in a clinical setting following balloon angioplasty. Antisense to PCNA inhibits rat arterial SMC proliferation *in vitro* (Speir *et al.* 1992) as well as being able to inhibit the intimal hyperplasia in a balloon-injured rat carotid artery after short term extraluminal delivery (Simons *et al.* 1994). As of yet, there have been no reports of antisense oligonucleotides being used in a clinical setting.

2 f) CONCLUSIONS

Much remains unknown about the process of intimal hyperplasia, particularly as it relates to the development of vein graft stenoses. However, intimal hyperplasia appears to be a response to injury in blood vessels. The endothelium, the smooth muscle cells, circulating macrophages and platelets are all able to liberate growth factors which can stimulate SMC migration and proliferation. The principal growth factors in most models appear to be Platelet-Derived Growth Factor (PDGF), basic Fibroblast Growth Factor (bFGF) and more recently Endothelin-1 (ET-1). There are variations in the response of tissues from different animals to various growth-promoting stimuli and there are variations within the same animal between cells derived from the arterial tree and those from the venous system. These differences are particularly noticeable in the responses to flow. Venous SMCs appear to be stimulated to a greater extent by cyclic stretching and in some animals venous, but not arterial, endothelial cells alter their alignment in response to applied shear. In general, reduced shear and increased cyclic stretch promote SMC proliferation whilst increased shear and decreased cyclic stretch reduce intimal hyperplasia. Whilst several substances may influence intimal hyperplasia in animal and tissue culture models, there is as yet no drug which has been convincingly shown to inhibit intimal hyperplasia in humans (Clowes 1991a).

CHAPTER 3 Introduction to Cell and Organ Culture work

3 a) **INTRODUCTION**

3 b) **CULTURE OF SMCS**

i) ENZYME-DISPERSION METHOD

ii) MEDIAL EXPLANT TECHNIQUES

3 c) **ORGAN CULTURE**

i) HISTORY OF ORGAN CULTURE

ii) ORGAN CULTURE OF ARTERIAL TISSUE

iii) ORGAN CULTURE OF HUMAN SAPHENOUS VEIN

3 a) INTRODUCTION

Animal models of intimal hyperplasia are useful but the responses of various laboratory animals to vascular injury vary considerably between species (Ferrell *et al.* 1992) and do not always apply to man. One way to get around this problem is to use cultured human tissue. Whilst this has limitations compared to in situ tissue in a living animal, it at least has the advantage of being human tissue. Cell culture allows the study of individual vascular cell types. This has the drawback that isolated cells in culture are unlikely to act in the same way as cells *in vivo*, where they have a different extracellular environment and intricate interrelationships with other cell types. In addition, cell culture tends to select out a clone or clones of cells which are particularly suited to the culture conditions. These cells may not necessarily represent the cell type as a whole or indeed represent those cells which contribute to the disease process. However, cell culture does enable study of the ways in which a particular cell type reacts to various stimuli. In the study of vascular disease, this can provide information on the contribution of different cell types to the process of intimal hyperplasia. In addition to culturing individual cell lines, it is also possible to culture a combination of vascular tissues which maintain their normal cellular relationships with other cell types. This organ culture method is more closely related to the *in vivo* situation.

3 b) CELL CULTURE

The basic pathology underlying intimal hyperplasia is a proliferation of the vascular smooth muscle cells (SMCs). Any attempt to understand the process of intimal hyperplasia must include an attempt to understand the processes involved in migration and proliferation of the vascular SMCs.

Smooth muscle cells were first cultured as far back as 1913 (Champy 1913). Smooth muscle cells may be obtained from intact blood vessels using an enzyme dispersion method, free-floating explants or substrate-attached explants (Chamley-Campbell *et al.* 1979).

3 b.i) ENZYME-DISPERSION TECHNIQUES

Enzyme-dispersion is used to obtain smooth muscle cells in a well differentiated contractile state. The vein is initially chopped into 1-2mm^3 segments and then placed into a physiological solution (e.g. Hanks Balanced Salt Solution) with 0.1% collagenase. This helps break down the extracellular matrix, and is followed by the addition of 0.05-0.1% Trypsin to help disperse the cells. Suspensions of cells in trypsin can then be added to foetal calf serum to neutralise the trypsin, before being centrifuged and resuspended in culture medium. Other useful proteolytic enzymes include hyaluronidase and elastase (Chamley-Campbell *et al.* 1979). The disadvantage of using enzyme dispersion is the theoretical damage to the SMCs or to surface receptors on the SMCs by the proteolytic enzymes used. This could alter the responses of the SMCs to various stimuli.

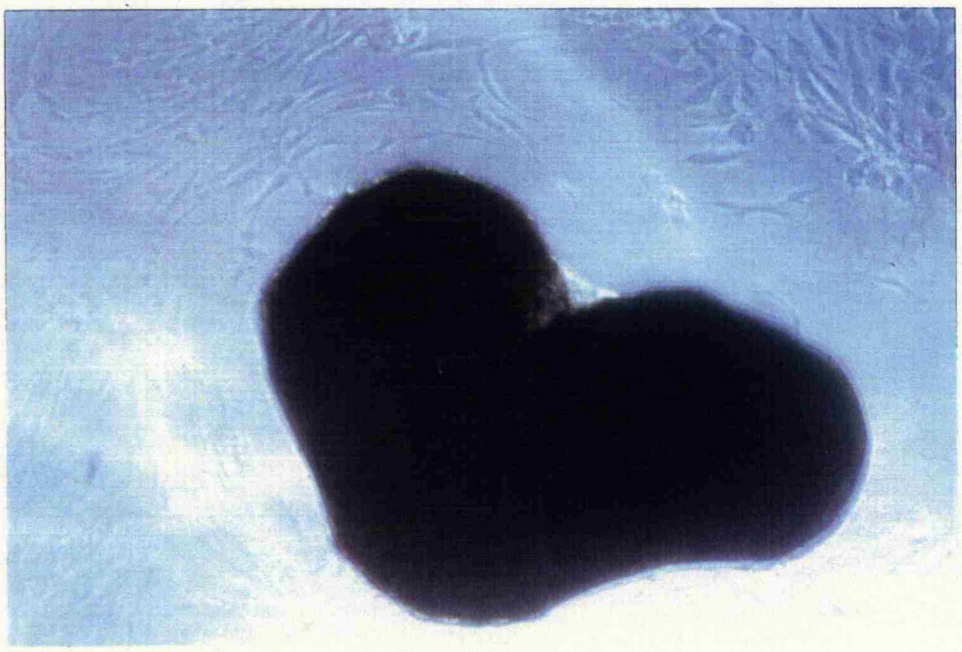
3 b.ii) EXPLANT TECHNIQUES

Free floating explants involve the stripping of the adventitia from the tissue and the placement of small (1 x 1mm-1 x 5mm) segments into culture media. There needs to be sufficient media to prevent the explants from adhering to the underlying substrate. The SMCs do not grow out from the explants but can be studied within the explants.

A more usual way to study explants of SMCs is to place the explants in just enough medium to keep them moist but not enough to allow them to float. The explants can then adhere to the substrate and eventually SMCs will migrate out (Figure 3b.1). These SMCs can then be subcultured by removing the medium, washing with a physiological salt solution and incubating with 0.05% trypsin at 37°C for 10 minutes. The cells can be grown to confluence and repassaged again up to 9 times (Chamley-Campbell *et al.* 1979). This method has the advantage of having minimal enzymatic degradation of the SMCs (trypsin is only used for long enough to separate the SMCs from the surface of the substrate). However, it does have the disadvantage that it will preferentially select out those cells that will migrate out of the explants and these cells may not necessarily reflect the majority of the cells in the blood vessel. The method may well lead to those cells with the greatest mitogenic potential being selected. In

addition, those cells which do migrate out from the explants are more likely to be of a secretory than a contractile phenotype. While this reflects the state of SMCs most found in intimal hyperplasia, it is not representative of cells found in the intact vessel, where most are in the contractile phenotype.

Figure 3b.1 Human saphenous vein explant x40. Explants of human saphenous vein with SMCs growing out after 2 weeks of culture.



Smooth muscle cells grow best in a medium containing 5-15% serum (Chamley-Campbell *et al.* 1981a, Blank *et al.* 1990, Aoyagi *et al.* 1991). Foetal calf serum (FCS) is the most widely used serum for cell culture. If grown in unsupplemented medium without serum, SMCs lose protein and DNA and become non-viable. They can, however be maintained in a quiescent state in the presence of a well-defined medium containing essential nutrients such as insulin, ascorbate and transferrin (Libby *et al.* 1983). SMCs tend to form a characteristic 'hill and valley' pattern in culture (Figure 3b.2). The 'hills' represent a confluence of cells which may be up to 10-15 layers thick whilst the sparse 'valleys' may represent only 1-3 layers (Chamley-Campbell *et al.* 1979). This characteristic pattern helps to distinguish SMCs from fibroblasts which may contaminate cell cultures, particularly if the adventitia has not been completely removed from the vascular tissue prior to harvest of the SMCs. Smooth muscle cells are also

distinguished from fibroblasts by their ability to stain positive for alpha actin, even in their secretory phenotype (Figure 3b.3).

Figure 3b.2 Human saphenous vein SMCs in culture. The SMCs show a typical 'Hill and Valley' pattern in culture.

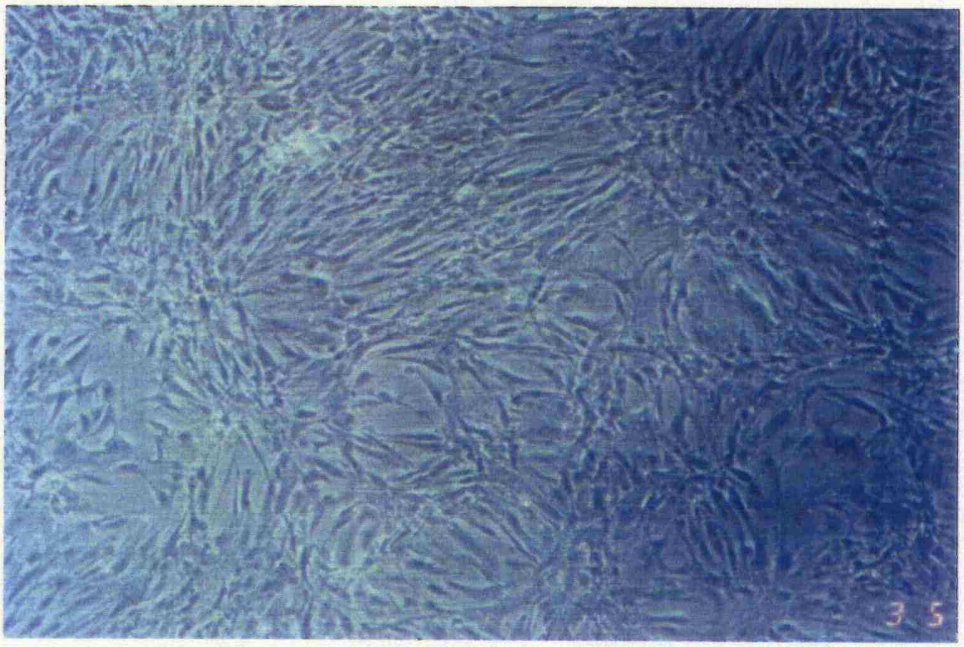
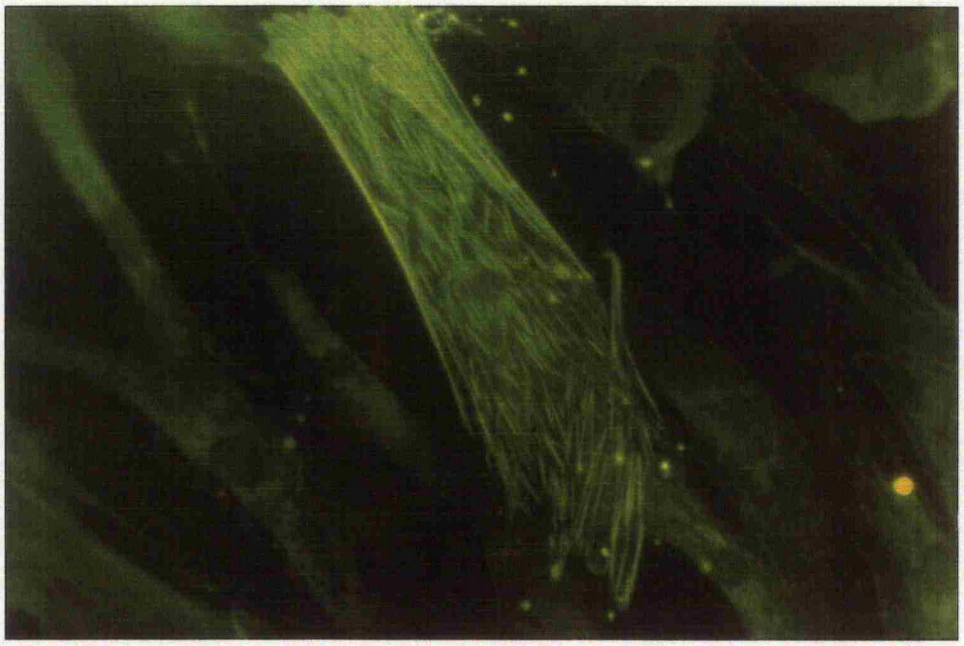


Figure 3b.3 Human saphenous vein SMCs stained with a monoclonal antibody to smooth muscle actin. The stain attaches to the actin and causes it to fluoresce bright green.



3 c) ORGAN CULTURE

In the *in vivo* situation, SMCs in the media are in close contact with endothelial cells (ECs). This condition can be recreated by using a bilayer coculture system (Graham *et al.* 1991, Xu *et al.* 1993, Gallicchio *et al.* 1994).Such a system allows some interaction between SMCs and ECs and more closely approximates *in vivo* conditions.

Another way to maintain the relationship between the endothelium and the smooth muscle layer of the media is to culture the vessel as a whole in culture medium. The viability of the tissue can be maintained in this organ culture as nutrients have a relatively short distance to diffuse.

The first reported case of vascular tissue being maintained in organ culture was by Trowell (Trowell 1959). He found that he could maintain small ring segments of rat artery for 9 days in culture with a normal histological appearance at the end of this period.

Barrett et al. cultured human thoracic aortic segments, obtained at autopsy, for up to 18 weeks in serum-supplemented medium (Barrett *et al.* 1979). They found a substantial accumulation of smooth muscle cells and extracellular matrix within the aortic intima after 1 week of culture. The intimal SMCs were also described as being more rounded, containing large golgi complexes and rough endoplasmic reticulum, features consistent with a secretory phenotype. After 30 days, the medial SMCs began to migrate out of the explant as a band and grow around the explant. At 18 weeks, the surface of the explant was covered in several layers of SMCs but most of the cells within the media were no longer viable. This proliferation was only seen in the presence of 5% FCS. If serum was not added to the culture medium then no proliferation occurred. As this technique relied on obtaining tissue from post mortems under diverse conditions, the endothelial coverage on the aorta was variable.

Pederson and Bowyer used segments of rabbit aorta in organ culture to assess the effects of controlled injury to the endothelium (Pederson *et al.* 1985). They ensured that the

segments of aorta harvested from the rabbit had an intact endothelium, as assessed by electron microscopy and the ability to exclude trypan blue staining. Endothelial integrity was maintained for up to 6 weeks in culture when the medium was supplemented with 30% FCS. At lower concentrations, the endothelium became fragmented. Experimentally defined superficial injuries to the endothelium were healed by migration of endothelial cells over the wound. There was no proliferation or migration of medial SMCs in this model.

Fingerle et al. used a chinchilla rabbit aorta for their organ culture model (Fingerle *et al.* 1987). This animal's aorta contains SMCs in the intima under normal conditions, as does that of humans. Their tissue samples remained viable for up to 28 days in culture, and indeed showed no loss of contractility at up to 16 days, when supplemented with 30% FCS. There was no change in the proliferation index (the percentage of cells proliferating) in either the intima or the media of the aorta at 14 days in unmanipulated aorta segments. Removal of the endothelial cells did not stimulate proliferation whilst a mechanical injury to the media did lead to an increase in intimal SMC numbers and an increase in intimal thickness.

Koo and Gotlieb studied the interaction of endothelial cells and intimal SMCs in a porcine aortic organ culture system (Koo *et al.* 1989). This animal also has intimal SMCs in the aorta under normal conditions. Koo and Gotlieb found that after 7 days culture in a medium supplemented with only 5% FCS, this model developed significant hyperplasia of the intimal SMCs. This SMC proliferation could be reduced by an initial gentle denudation of the aortic media, implying that an intact endothelial cell layer is required for this SMC growth. Conditioned medium was collected from the aortic cultures with intact endothelium. This medium was subsequently able to stimulate proliferation in denuded aortae and also in isolated cultured porcine SMCs. They concluded from these experiments that endothelium was able to stimulate intimal hyperplasia, at least in their organ culture model.

Whilst aortic organ cultures are useful as a model of intimal hyperplasia in arteries, they may not reflect the mechanisms of intimal hyperplasia in saphenous vein, the conduit of choice in infrainguinal bypass grafting in man. Soyombo et al. were the first group to describe organ culture of human saphenous vein (Soyombo *et al.* 1990). They showed that segments of human

saphenous vein could be cultured for 14 days in medium supplemented with 30% FCS. They assessed tissue viability by measuring adenosine triphosphate (ATP) levels. These decreased by only 20% over 14 days. A neointimal layer which stained positive for smooth muscle actin was present at the end of the 14 day culture period. Only about half of the intimal SMCs took up tritiated thymidine, suggesting that the remainder had migrated there from the media and were not actively proliferating. Surgical preparation of the vein during harvesting had no effect on intimal proliferation but did increase medial proliferation in this model, probably as a result of the release of mitogens from damaged medial SMCs (Angelini *et al.* 1991, Soyombo *et al.* 1993a). Removal of the endothelium, as with the porcine aortic organ culture model of Koo and Gotlieb, led to a reduction in intimal proliferation.

Holt et al. compared the responses of saphenous vein and internal mammary artery (IMA) in organ culture (Holt *et al.* 1993). They used both 'freshly-isolated' and 'surgically-prepared' saphenous vein in their comparison. The freshly-isolated vein had minimal endothelial denudation or medial damage whilst the surgically-prepared vein had partial endothelial denudation and some medial damage. The IMA sustained minimal damage during harvesting and had an intact endothelial layer. After 14 days of culture, all 3 organ cultures showed intimal proliferation. However, the surgically-prepared segments of vein showed significantly more proliferation than the freshly-isolated vein or the IMA which were very similar in their degree of intimal hyperplasia. The authors concluded that damage to the vein during surgical preparation may partially explain the difference in patency between saphenous vein and IMA aortocoronary bypass grafts. It is usual for the saphenous vein to be distended during harvesting for use in aortocoronary bypass surgery whilst the IMA undergoes minimal handling and is not distended.

Soyombo et al. further examined the control of SMC proliferation by the endothelium in the saphenous vein organ culture (Soyombo *et al.* 1993b). They again demonstrated an inhibitory effect on proliferation by gently denuding the endothelium. In-situ hybridisation techniques demonstrated both PDGF-A and -B chains in the venous endothelium prior to culture of intact segments of vein. Expression of both of these chains increased during culture and they were also found in the intimal SMCs. Immunocytochemistry with antibodies to bFGF

showed that this growth factor was largely confined to the endothelium prior to culture and the labelling became less intense with culture. The addition of antiserum to bFGF for the first 3-4 days of culture led to a small but significant reduction in the thymidine index in the intima of surgically-prepared veins, though it had no effect on freshly-isolated veins, implying a role for bFGF only where there is significant cell damage. The effect of the vasodilators EDRF and prostacyclin were examined indirectly in the same paper and again in a further publication (Soyombo *et al.* 1995). As these mediators are labile in cell culture, the 8-Bromo analogues of cyclic adenosine monophosphate (cAMP) (which mediates the action of prostacyclin) and cyclic guanosine monophosphate (cGMP) (which mediates the action of EDRF) were studied. Both nucleotides reduced intimal thickening and reduced the number of thymidine labelled cells. 8-Bromo-cAMP was the most effective at the same concentration, and also reduced the number of proliferating medial SMCs.

Studies in our own department have also looked at the importance of the endothelium in intimal hyperplasia in the organ culture model of human saphenous vein. Allen et al. demonstrated that whilst an intact segment of vein developed a neointima in culture and a segment denuded of its endothelium did not, a denuded segment of vein cocultured in the same petri dish as an intact vein did develop intimal hyperplasia (Allen *et al.* 1994). The denuded segment of vein in the coculture showed significantly more proliferation than a denuded vein by itself, but significantly less proliferation than an intact vein. This suggests a paracrine mediator produced by the intact endothelium and released into the culture medium, an effect similar to the conditioned medium from Koo and Gotlieb's porcine aortic culture (Koo *et al.* 1989).

Organ culture of human saphenous vein is a useful model with which to study the effects of various drugs on SMC proliferation. Francis et al. looked at the effect of heparin in this model (Francis *et al.* 1992). Whilst heparin has been shown to have an antiproliferative and an antimigratory effect on SMCs in some animal (Clowes *et al.* 1977, Dryjski *et al.* 1988, Wilson *et al.* 1991) and cell culture models (Painter 1991a, Gilbertson *et al.* 1992, Yamaguchi *et al.* 1993), there was no inhibition of neointimal formation in this experiment. The authors used relatively high concentrations of heparin with no effect and concluded that the SMCs in

human saphenous vein were probably less responsive to heparin than SMCs in rat aorta. A vein culture study from our own department did show a significant antiproliferative effect with a high dose of low molecular weight heparin (Varty *et al.* 1994b), despite the fact that Chan et al. found that the antiproliferative effect of heparin on cultured human saphenous vein SMCs was more pronounced in the higher molecular weight fractions (Chan *et al.* 1992). The neointimal thickness in the vein culture experiment was reduced from 21μm to 7μm. As the dose required was higher than what could safely be used as a serum level *in vivo*, the authors concluded that a non-anticoagulant fraction of heparin might be of benefit in inhibiting intimal hyperplasia. Another paper from our department looked at the effect of the non-peptide angiotensin II receptor antagonist losartan in the human saphenous vein organ culture (Varty *et al.* 1994a). Angiotensin II has been shown to be a mitogen for SMCs (Naftilan 1992) and angiotensin-converting enzyme inhibitors (ACE Inhibitors) have had an antiproliferative effect in some animal models of intimal hyperplasia (Clowes *et al.* 1991b, O'Donohoe *et al.* 1991, Powell *et al.* 1991). In this experiment, losartan was able to significantly reduce both the neointimal thickness and the proliferation index after 14 days of culture.

Cell and organ culture models are a useful adjunct in the study of intimal hyperplasia. It is easier to accurately control the extracellular conditions than it is in *in vivo* experiments. Thus experimental conditions can be repeatable. In addition, it is possible to study human tissue in conditions which would not be possible *in vivo*. Samples of human tissue can be obtained relatively easily from patients undergoing aortocoronary or peripheral arterial bypass surgery. Paired experiments using one or more segments as a control can be performed with the tissue obtained. This is particularly useful in man as there is a greater variation between individuals than there is between laboratory animals. In particular, pre-existing intimal thickening and medial longitudinal hypertrophy are prevalent in patients undergoing arterial bypass surgery (Varty *et al.* 1995). The effects of this pre-existing disease both on the intimal hyperplasia developed in organ culture and on the formation of vein graft stenoses are unknown.

Figure 3c.1 Organ culture of human saphenous vein. The vein is placed on a polyester mesh and pinned into the sylgard resin gel using A1 minuten pins.

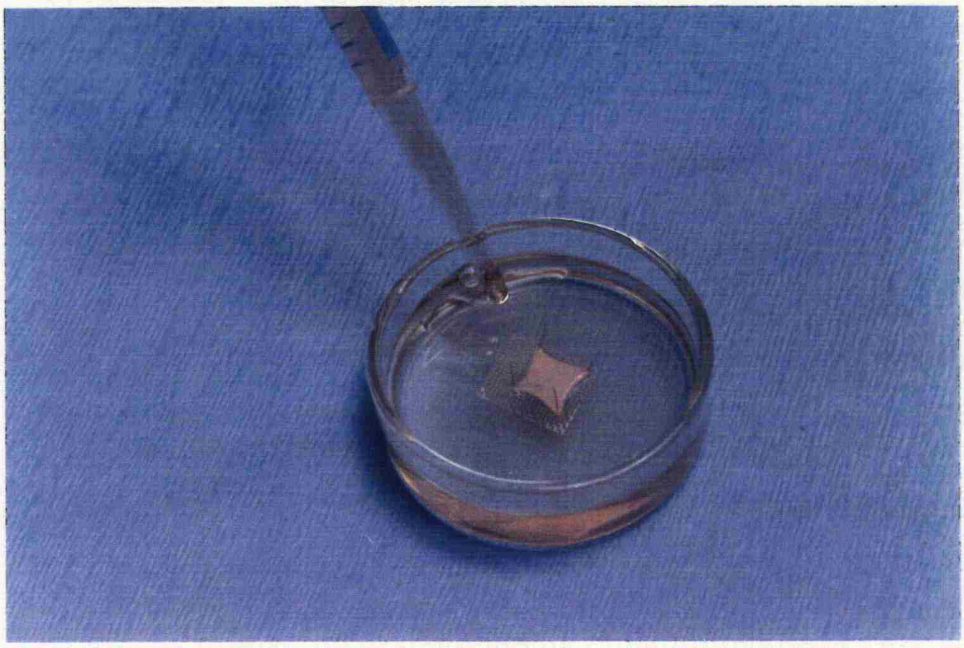
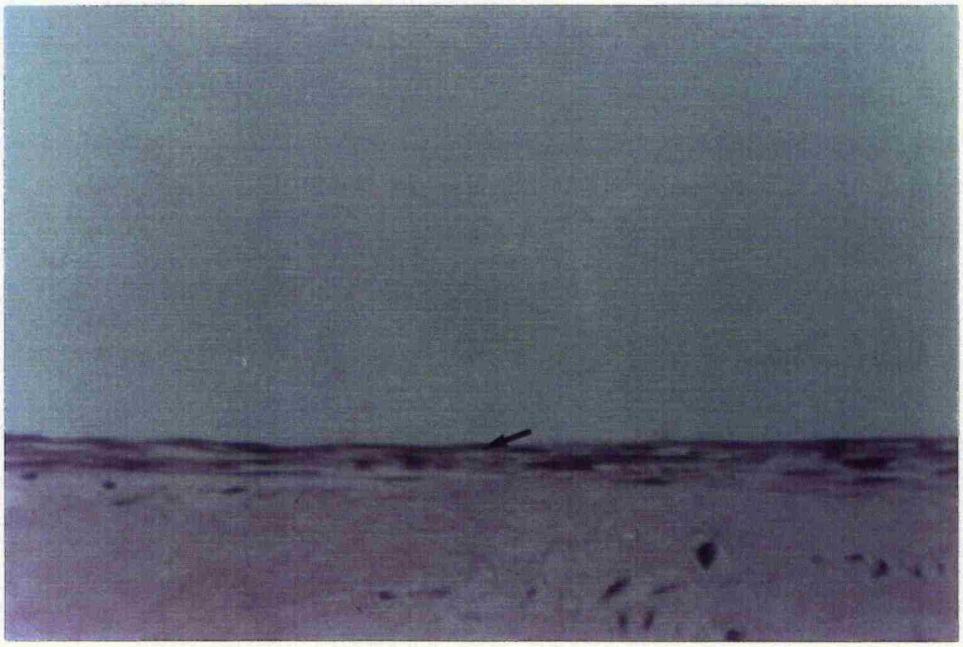


Figure 3c.2 Cultured human saphenous vein x 200 (Haemotoxylin + Eosin) at 14 days. The neointima developed by the cultured saphenous vein is shown by the arrow.



CHAPTER 4 -Review of management of venous and prosthetic infrainguinal bypass grafts at Leicester Royal Infirmary

4 a) THE LONG-TERM OUTCOME OF INFRAINGUINAL VEIN GRAFT SURVEILLANCE

i) INTRODUCTION

ii) METHODS

iii) RESULTS

iv) DISCUSSION

4 b) PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY OF INFRAINGUINAL VEIN GRAFT STENOSES: THE LONG-TERM OUTCOME

i) INTRODUCTION

ii) METHODS

iii) RESULTS

iv) DISCUSSION

4 c) THE FATE OF INFRAINGUINAL PTFE GRAFTS AND AN ANALYSIS OF FACTORS AFFECTING THEIR OUTCOME

i) INTRODUCTION

ii) METHODS

iii) RESULTS

iv) DISCUSSION

4 d) THE EFFECT OF A SURVEILLANCE PROGRAMME ON THE PATENCY OF SYNTHETIC INFRAINGUINAL BYPASS GRAFTS

i) INTRODUCTION

ii) METHODS

iii) RESULTS

iv) DISCUSSION

4 a) THE LONG-TERM OUTCOME OF INFRAINGUINAL VEIN GRAFT SURVEILLANCE

4 a.i) INTRODUCTION

Vein graft surveillance, as mentioned in Chapter 1, is of proven benefit in improving graft patency at least in the first year after arterial bypass surgery. However, there have been very few studies of the long-term effect of surveillance. The aim of this study was to look at the longer term outcome of the Leicester Royal Infirmary vein graft surveillance programme on graft patency. In a previous publication from our department, London et al. reviewed 112 consecutive vein grafts followed up in this surveillance programme for a median period of 14 months (London *et al.* 1993). These grafts had a primary patency of 40% at 42 months with primary assisted and secondary patencies of 65% and 69% respectively. Those patients who remain alive with patent grafts continue to be followed up in the graft surveillance programme. This study describes the longer term outcome of these grafts.

4 a.ii) METHODS

All infrainguinal vein bypass grafts performed between July 1988 and March 1992 were entered into a vein graft surveillance programme (Brennan *et al.* 1991). There were a total of 112 vein grafts in 106 patients. The patients were examined in the vascular studies unit at intervals of 1, 3, 6, 9 and 12 months after their surgery then at 6 monthly intervals. A clinical assessment, measurement of resting and post-exercise ankle brachial pressure indices (ABPI), and duplex scan of the graft took place at each visit. Since November 1991, the graft and tibial vessels have also been routinely insonated with a colour-coded duplex scanner (Diasonics Spectra, Diasonics Sonotron, Bedford, U.K.). Arteriography was performed if there was a serial fall in the resting ABPI >0.1 or a post-exercise fall in ABPI >0.1 or a segmental peak velocity ratio increase >2.5 on duplex.

The initial treatment for any vein graft stenoses detected by surveillance was percutaneous transluminal angioplasty (PTA). Only one stenosis was treated primarily with

open revision. This was for a long diffuse narrowing in the distal part of a below-knee popliteal in-situ graft in a 70 year old lady occurring 5 months after surgery.

Graft patency was assessed using the recommendations of Rutherford (Rutherford 1991) based on the suggestions of the Ad Hoc Committee on Reporting Standards of the Society for Vascular Surgery (Rutherford *et al.* 1986). Primary patency was defined as uninterrupted patency after the initial surgery with no further procedure performed on the graft. Primary assisted patency requires that the graft remains patent but procedures such as PTA can be performed to prevent subsequent graft failure. Secondary patency allows procedures to be performed to salvage an occluded graft. Patients who died during follow up were censored to their last clinic attendance.

Actuarial patency curves were computed using the Kaplan-Meier method (Kaplan *et al.* 1958) and compared with the Mantel-Haenszel log-rank test (Mantel *et al.* 1959). Univariate analysis using the log-rank test was used to identify factors affecting secondary graft patency. Variables with a $p \leq 0.05$ were entered into a Cox proportional hazard model using the forward stepwise entry method (Cox 1972). Variable entry was terminated when the improvement in the model was not statistically significant ($p=0.05$). Statistical analysis was performed using the statistical package SPSS for Windows (SPSS, Chertsey, U.K.).

4 a.iii) RESULTS

The median [range] age of the patients at operation was 75 [32-97]. The male : female ratio was 68:38, there were 37 (35%) diabetics, 25 (24%) patients had a previous history of ischaemic heart disease, 33 (31%) patients admitted to continued smoking postoperatively and 43 (41%) patients were on aspirin postoperatively.

The 30 day mortality rate among the 106 patients undergoing surgery was 7%. Twenty-three (20%) of the grafts occluded during this period, 4 of these within the first 24 hours. The median [range] follow up for all patients was 34 [1-76] months. Twenty-eight patients (26%) died during follow up with a 30% mortality rate at 4 years. Twenty-two limbs

(20%) were lost with a 4 year limb salvage rate of 79%. Thirty-eight grafts (34%) developed at least one stenosis during follow up. The graft details are shown in Table 4a.1. The sites of the stenoses are shown in Table 4a.2. The median [range] interval to development of a stenosis was 5 [2-46] months. Eight primary stenoses occurred over 1 year after the initial operation. These stenoses had not been present at earlier attendances at the surveillance clinic.

Eight (7%) grafts required PTA to an inflow vessel at a median [range] interval of 15 [1-38] months after surgery, and 12 (11%) grafts required PTA of a run-off vessel at a median [range] interval of 31 [2-61] months after surgery. The presence of significant inflow or run-off disease was detected by the surveillance programme.

The patency, limb salvage and mortality curves are shown in Figures 4a.1 and 4a.2.

Table 4a.1 Details of the indications for the graft and the type of graft performed

Indication	
Claudication	15 (13%)
Rest pain	38 (34%)
Tissue necrosis	59 (53%)
Graft type	
In situ	79 (71%)
Reversed	33 (29%)
Graft Position	
Above-knee popliteal	6 (5%)
Below-knee popliteal	30 (27%)
Distal	76 (68%)

Table 4a.2 Sites at which the stenoses occurred

Site of stenosis	Number
Proximal anastomosis	3
Distal anastomosis	12
Proximal graft	6
Mid graft	7
Distal graft	10

Figure 4a.1 Patency curves showing primary, primary assisted and secondary patencies. The numbers at risk are shown at the bottom. The percentage patency is shown for each curve at 12, 24, 36 and 48 months. The standard error is less than 10% at all points. The primary assisted patency was significantly better than the primary patency (p=0.0001 Log Rank test).

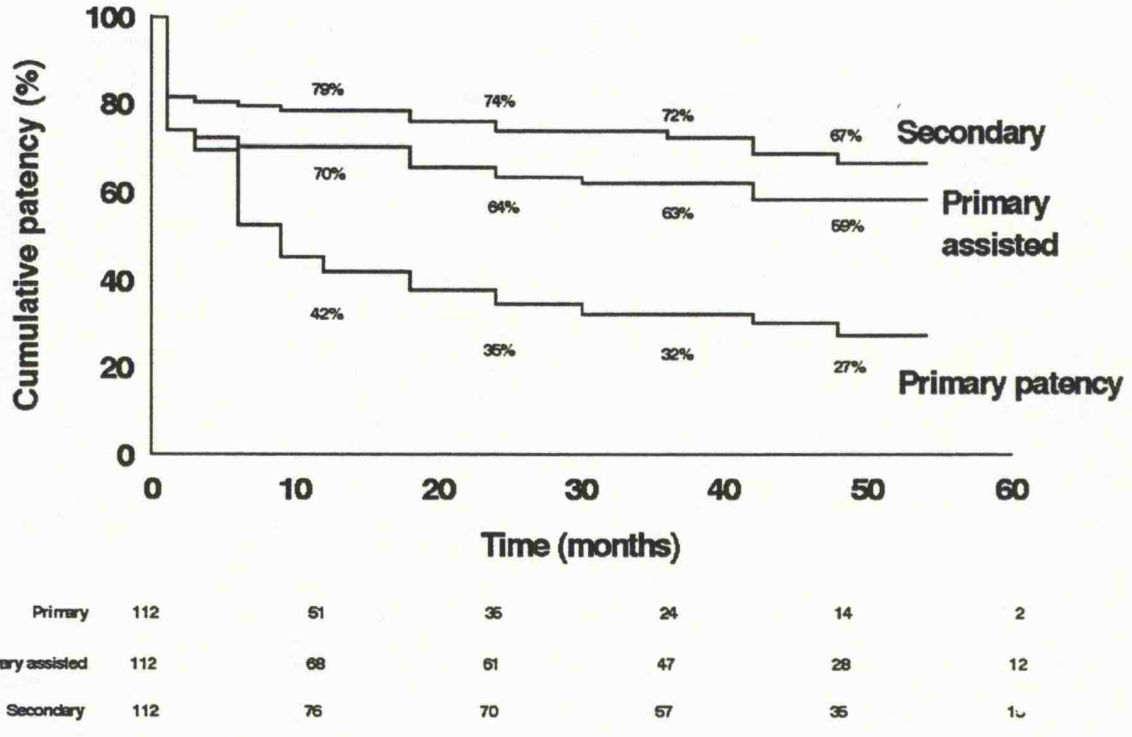
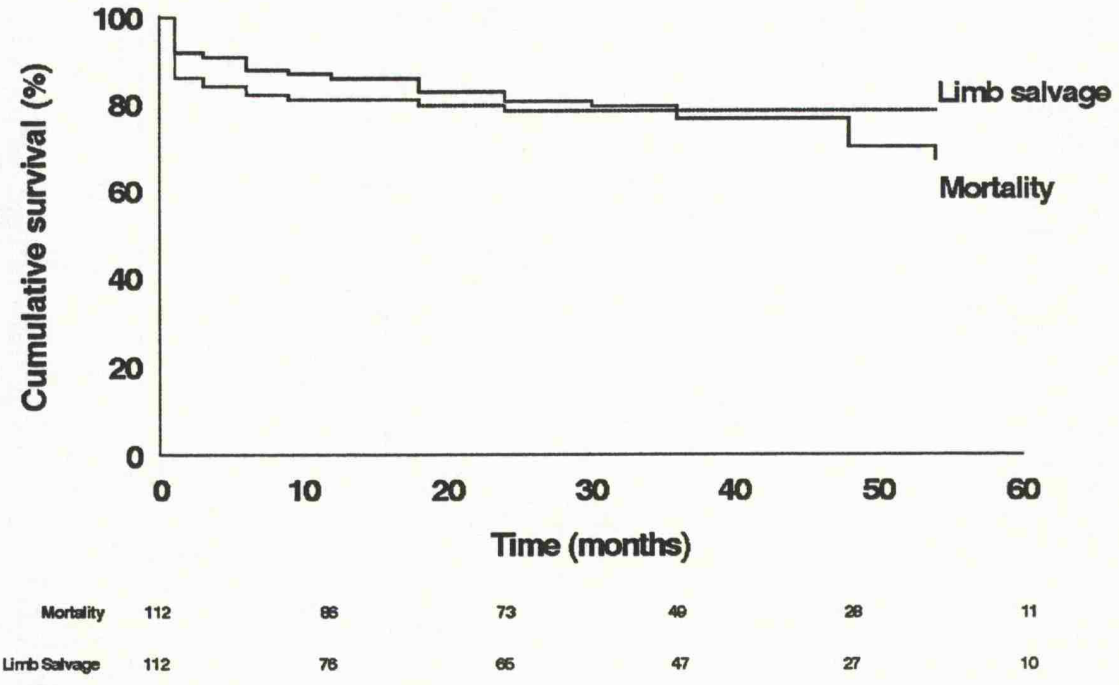


Figure 4 a.2 Limb salvage and patient survival curves for patients in surveillance programme. The numbers at risk are shown at the bottom. The standard error is less than 10% at all time points.



Neither primary, primary assisted nor secondary patency were adversely affected by the presence of diabetes mellitus, hypertension, a single vessel run-off or by not ingesting aspirin, using univariate analysis. There was a statistical increase in mortality in those patients with diabetes mellitus (p=0.006, Log Rank test), a single vessel run-off (p=0.0001, Log Rank test), those patients not taking aspirin (p=0.006, Log Rank test) and in patients of female gender (p=0.03, Log Rank test). Limb salvage was also statistically worse in diabetic patients (p=0.02, Log Rank test).

Multivariate analysis confirmed that none of the risk factors adversely affected primary, primary assisted or secondary patency. The presence of single vessel run-off (p=0.04, Odds ratio 3.4), female gender (p=0.04, Odds ratio 1.6) and not ingesting aspirin (p=0.004, Odds ratio 2) remained significant risk factors for mortality. The effect of diabetes mellitus on limb salvage was not significant (p=0.72) in a multivariate analysis.

4 a.iv) DISCUSSION

There are several reports in the literature strongly suggesting a benefit of surveillance in maintaining vein graft patency (O'Mara *et al.* 1981, Cohen *et al.* 1986, Moody *et al.* 1990, Mills *et al.* 1990, Harris 1992, London *et al.* 1993), and there has been one recent randomised control trial showing a substantial gain for vein grafts in a surveillance programme (Lundell *et al.* 1995). The recommended duration of a surveillance programme is uncertain. The time course of vein graft intimal hyperplasia in humans is not as well worked out as it is in animals. Angelini et al. showed that in experimental vein grafts in pigs, there was an initial rapid increase in medial and intimal smooth muscle cells in the first week, followed by a rapid increase in matrix production up to 4 weeks after surgery (Angelini *et al.* 1992b). After this time, there was a slow but gradual increase in smooth muscle cell numbers. Intimal hyperplasia in human vein grafts is thought to be maximal in the first 6 months after surgery, with few new stenoses after the first year. Some authors have reported very few remediable problems after the first year (Berkowitz *et al.* 1989, Brennan *et al.* 1991, Idu *et al.* 1992) and Taylor et al. reported no problems after the first year (Taylor *et al.* 1990), recommending that surveillance should be curtailed after one year on the basis of cost. However, whilst intimal hyperplasia is relatively uncommon over 12-18 months after bypass surgery, atherosclerosis is quite common (Dilley *et al.* 1988). Atherosclerotic plaques can develop within the vein graft or in the native arteries of the patient. If there is significant disease in an inflow or run-off vessel, this can be picked up by vein graft surveillance. In this study, there were eight first-time stenoses occurring over 1 year after bypass surgery. As these stenoses were not excised, the histology is not known for certain but they may well have been atherosclerotic. In addition, there were 8 inflow and 12 run-off problems requiring treatment. These problems in the native vessels were due to the progression of atherosclerosis. Five of the inflow and 8 of the outflow problems were found over 12 months after the initial operation. These problems would not have been found or treated had the patients not attended the graft surveillance clinic.

Most stenoses will go on to cause graft occlusion (London *et al.* 1993) and as the results of salvage procedures on occluded grafts are so poor (Belkin *et al.* 1990), an aggressive

policy on management of graft stenoses is justified. Vein graft patency and limb salvage can be improved by an effective surveillance programme. This is beneficial to the patient and gratifying to the surgeon. Also in these cost-conscious times, it is important to prevent unnecessary amputations, as the estimated cost to the community of an amputation in 1992 was over £25,000 (Harris 1992). Therefore, despite the relatively low number of stenoses found after 1 year, graft surveillance is currently continued for life at 6 monthly intervals after the first year. A 6 month rather than a 3 month interval was chosen because of the relatively small number of stenoses seen after the first year. A longer interval was thought to be too risky as there was a significant chance of the graft occluding between surveillance appointments. As the surveillance programme is already in place, the cost of seeing another patient at 6 monthly intervals is relatively inexpensive (The cost is currently £124 for a single limb and £155 for a bilateral scan.) compared to the cost of an amputation.

It was perhaps a little surprising to find that none of the risk factors associated with atherosclerosis seemed to have an effect on primary, primary assisted or secondary patencies in this cohort of patients. However, mortality was significantly affected by the presence of single vessel run-off preoperatively, female gender and the non-use of aspirin. The presence of a single run-off vessel is an indication of severe lower limb atherosclerotic disease and is a reflection of atherosclerotic disease elsewhere, particularly the coronary arteries. Female gender appears to adversely affect mortality but this is related to the fact that women develop atherosclerotic disease at a greater age than men. The median [range] age for women in this group was 76 [37-85] whilst the median [range] age for men was 67 [37-97]. Aspirin has been shown to be beneficial in preventing cardiovascular morbidity and mortality (Sarin *et al.* 1993) and even if it does not improve graft patency, it may allow the patient to live longer with his patent graft. This in itself should be reason enough to recommend its routine use.

4 b) PERCUTANEOUS TRANSLUMINAL ANGIOPLASTY OF INFRAINGUINAL VEIN GRAFT STENOSES: THE LONG-TERM OUTCOME

4 b.i) INTRODUCTION

The aim of this prospective study was to examine the long-term outcome of treating infrainguinal vein graft stenoses by percutaneous transluminal angioplasty (PTA) as the first line treatment. Bypass surgery is frequently the treatment of choice for severe lower limb ischaemia. Although autologous vein is the best available conduit (Budd *et al.* 1990), 20-35% of infrainguinal vein grafts will develop patency-threatening stenoses (Szilagyi *et al.* 1973, Grigg *et al.* 1988b, Moody *et al.* 1989, London *et al.* 1993), the majority occurring within the first postoperative year. A previous review of 112 consecutive infrainguinal vein grafts (London *et al.* 1993) that had been entered into a graft surveillance programme found that 30 (27%) grafts developed stenoses. It has been the policy of our unit to treat these stenoses by percutaneous transluminal angioplasty (PTA) in the first instance and to reserve surgical revision for those cases in which PTA is unsuccessful. However, it has been argued that PTA is not a durable procedure and that surgical revision should be the preferred first line treatment for vein graft stenoses (Perler *et al.* 1990, Bandyk *et al.* 1991). The purpose of this prospective study therefore was to report and review the long-term outcome after PTA of vein graft stenoses.

A previously published study of 112 infrainguinal vein grafts performed at Leicester Royal Infirmary over a 44 month period (July 1988 to March 1992) found that 30 grafts in 29 patients developed stenoses at 33 sites (London *et al.* 1993). This study examines the long-term outcome in the 29 patients and 30 grafts previously reported.

4 b.ii) METHODS

Graft stenoses were detected by surveillance (Brennan *et al.* 1991) and treated in the first instance by PTA (or PTA and thrombolysis in the case of 2 grafts which presented with

occlusion). Angioplasty was performed antegradely using hydrophilic angled guidewires (Radiofocus; Terumo, Tokyo, Japan) with balloon catheters (Stellar 535; PSG, Mountain View, California, USA or Medi-Tech Ultra-Thin; Med-Tech, Watertown, Massachusetts, USA) of the same diameter as the graft. Stenoses were dilated for 30 seconds at a time using pressures up to 12 atmospheres, repeated up to 3 times as necessary. Patients were given 150mg aspirin orally prior to angioplasty. This was continued for 3 months after the angioplasty. A single bolus of 5000 units heparin was also given during the procedure. Intravenous tolazoline (12.5mg) was also given at the start and at the end of the procedure to reduce vascular spasm. After successful PTA, patients were re-entered into the graft surveillance programme and seen at 1, 3, 6, 9,12 months and 6 monthly thereafter. Ankle Brachial Pressure Indices (ABPI) were recorded before and after exercise, and a colour-coded duplex scan was performed of the graft, inflow and outflow tracts. Arteriography was requested if there was a serial fall in the resting ABPI of >0.10, a post-exercise fall >0.10 or if duplex scanning revealed a segmental peak velocity ratio increase of >2.5.

Actuarial patency curves were computed using the statistical package SPSS for Windows (SPSS, Chertsey, U.K.). The cumulative patency rates were compared using a log-rank test (Mantel *et al.* 1959).

4 b.iii) RESULTS

The median [range] follow up after the first angioplasty was 39 [18-56] months and 14 of 33 (42%) stenoses recurred at a median [range] interval of 8.5 [1-39] months. There was no difference in follow up between those patients who restenosed and those who did not (median follow up 35 and 43 months respectively, P=0.16 Mann-Whitney U test). Nine (30%) grafts developed a stenosis in at least one other site at a median interval of 17 (1-48) months after the first angioplasty. Because these stenoses are new events and not recurrences they are not considered further in the analysis. The details of the 33 stenoses treated in the first instance by PTA are shown in Table 4b.1. The subsequent outcome of the 14 recurrent stenoses is shown in Figure 4b.1. It can be seen that 8 (57%) recurrent stenoses were successfully treated by a second PTA, 5 (36%) required surgical revision and 1 (7%) was treated by a third PTA.

Table 4b.1 Details of the 33 stenoses, the grafts in which they occurred and subsequently recurred, and their initial length. The figures in parentheses are percentages.
*P=0.03 Log rank test for recurrences in reversed compared to in-situ grafts.

	Number of stenoses	Recurrence after first PTA
Graft type		
In-situ	24	*13 (54)
Reversed	9	1 (11)
Graft position		
Above knee popliteal	2	0 (0)
Below knee popliteal	10	3 (30)
Distal	21	11 (52)
Stenosis length		
<2cm	31	15 (48)
>2cm	2	0 (0)

The overall cumulative restenosis rate is shown in Figure 4b.2. Table 4b.2 gives the recurrence at each site along the graft. and the cumulative recurrence rates for different sites in the graft are shown in Figure 4b.3. Twelve (86%) of the recurrences were in the distal third of the graft (6 at the distal anastomosis), significantly higher than the rates in either the proximal or mid graft (P=0.002 Log Rank test). The cumulative recurrence rates in reversed (27%) and in situ (73%) grafts are shown in Figure 4b.4, these recurrence rates were significantly different (P=0.03 Log Rank test).

The cumulative limb salvage in the 30 limbs that contained graft stenoses is shown in Figure 4b.5. One patient lost a limb after a failed angioplasty followed by an unsuccessful graft revision (interposition vein graft).

Table 4b.2 Sites of original 33 stenoses and recurrence rate. The figures in parentheses are percentages. Two stenoses in the proximal part of the graft were are at the anastomosis. Nine stenoses in the distal graft were at the anastomosis.

*P=0.002 Log Rank test for cumulative recurrence rate in the distal graft compared to recurrences in the proximal or mid graft.

Site of original stenosis	Number	Recurrences (percentage)
Proximal graft	8	1 (13)
Mid graft	7	1 (14)
Distal graft	18	*12 (67)

Figure. 4b.1 Outcome of treating the 33 stenoses by PTA as first line treatment.

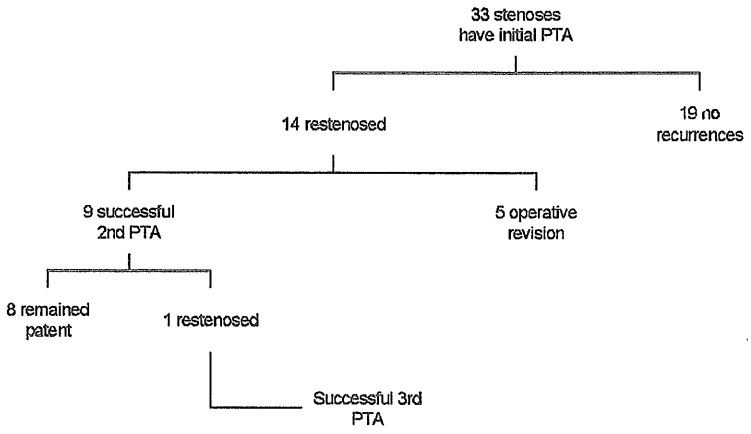


Figure. 4b.2 Overall cumulative restenosis rate after a single angioplasty.

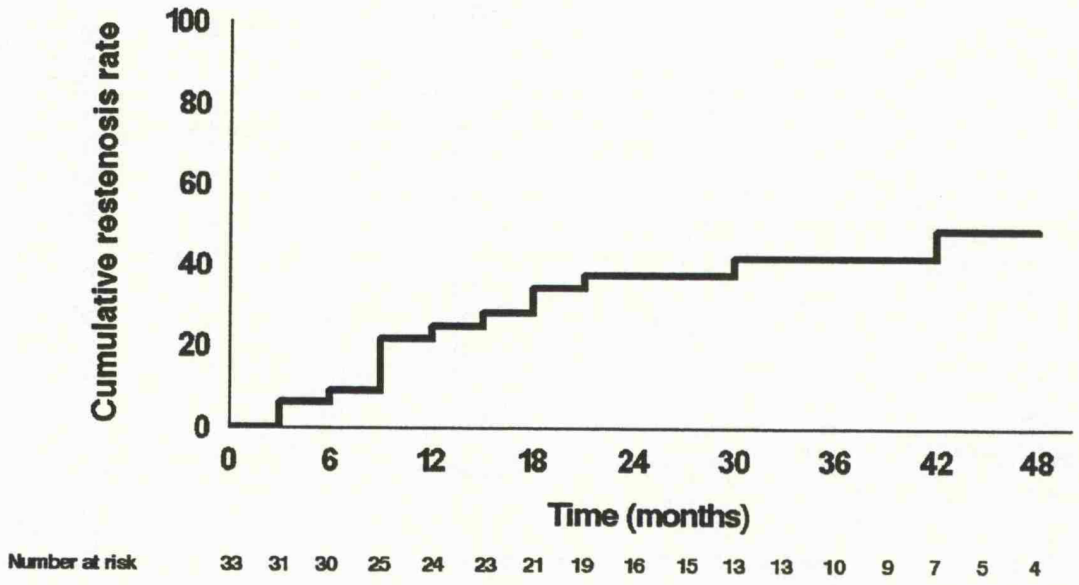


Figure. 4b.3 Cumulative restenosis rates for stenoses occurring in the proximal, middle and distal thirds of the graft. P=0.002 for equality of distribution of restenoses, Log rank test.

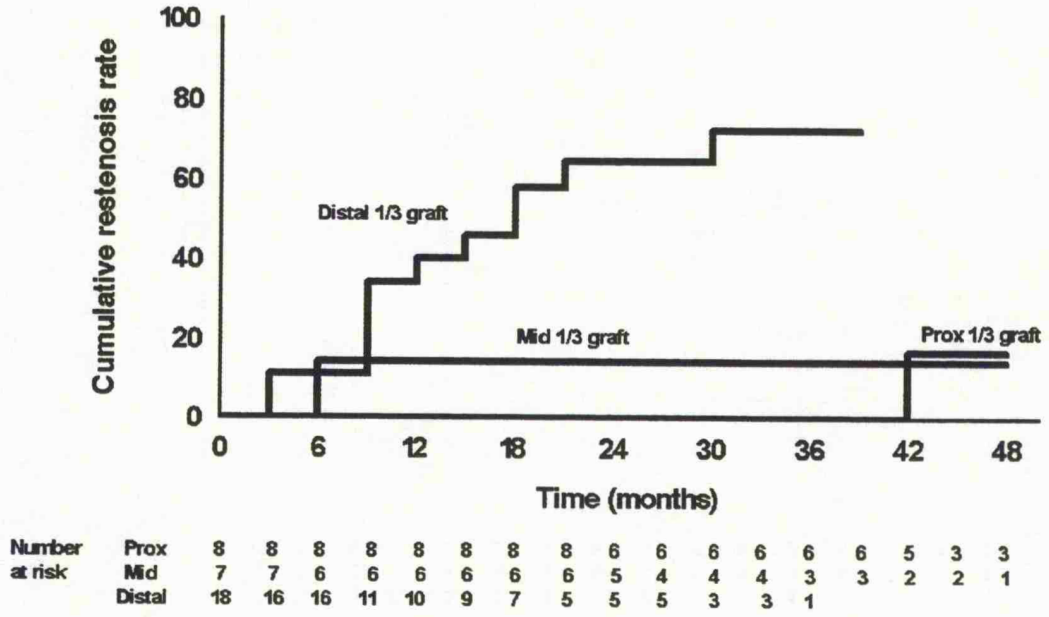


Figure. 4b.4 Cumulative restenosis rates for reversed and in situ grafts. P=0.03 for equality of distribution of restenoses, Log rank test.

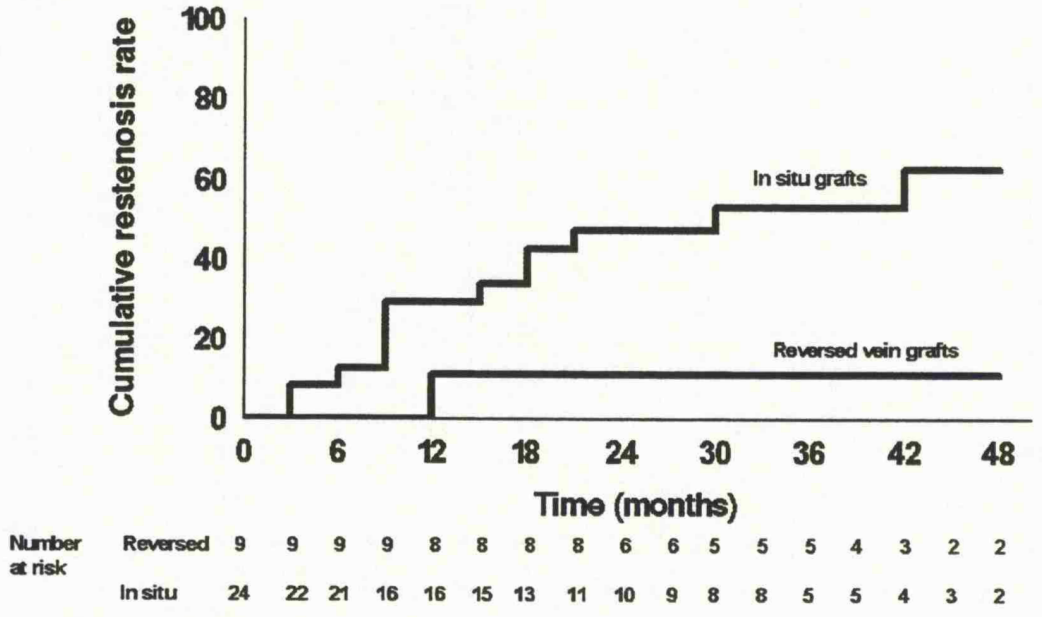
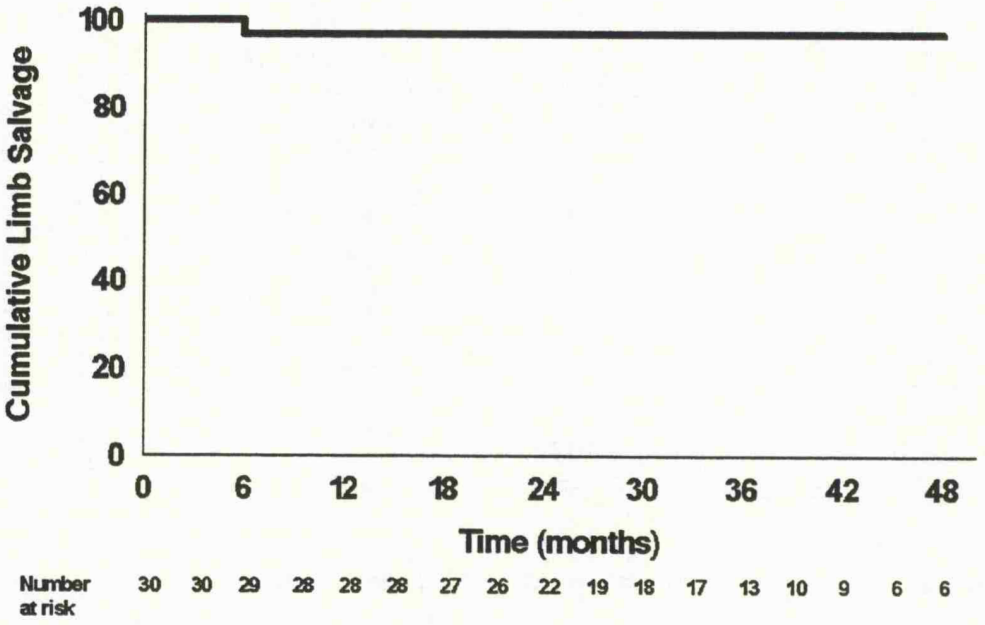


Figure. 4b.5 Cumulative limb salvage in the 30 limbs that contained the 33 stenoses.



4 b.iv) DISCUSSION

Although it has been shown that a surveillance programme can improve infrainguinal vein graft patency, there is debate concerning the optimal treatment of detected stenoses. The options are surgical revision, PTA or endovascular stenting. The reported experience with endovascular stenting in this situation is very limited (Davies *et al.* 1993a). Intravascular stenting has not been used for the treatment of vein graft stenoses in this study because of concerns about the flow-limiting effect of a stent in a low diameter vessel such as the distal section of an infrainguinal graft. Percutaneous Transluminal Angioplasty is used as the first line treatment in preference to surgery because it is relatively non-invasive and can be rapidly arranged and performed in our unit.

This study shows that whilst the majority (58%) of infrainguinal vein graft stenoses can be treated by a single PTA, that 42% of stenoses treated by PTA alone recur. Stenoses in the distal third of the graft and at the distal anastomosis were particularly likely to recur and in situ vein grafts were more likely to develop recurrent stenoses after angioplasty than reversed vein grafts. This is despite the fact that there is no statistical difference between reversed and in situ vein grafts with respect to the formation of the original stenosis (Harris *et al.* 1988, London *et al.* 1993). In situ vein grafts might be expected to have a higher incidence of restenosis at their distal end because this is where their diameter is smallest (Varty *et al.* 1993a) and small diameter vein is an independent risk factor for graft stenosis (Varty *et al.* 1993b). Berkowitz et al. (Berkowitz *et al.* 1992) found that most of the stenoses in a series of *reversed vein* grafts were in the proximal third, ie that part of the vein with the smallest diameter. Interestingly, in the present study only one of 8 stenoses in the proximal third of a graft recurred and this was in a reversed vein graft.

Several authors have questioned the durability of PTA of vein graft stenoses (Perler *et al.* 1990, Bandyk *et al.* 1991, Whittemore *et al.* 1991, Idu *et al.* 1992, Marin *et al.* 1993a). Patency was maintained by PTA alone in 23 (77%) of the grafts that developed stenoses and only 5 (17%) grafts needed an additional operative procedure to maintain the patency of the initial PTA site. However, the recurrence rate in the distal third of the graft was high, with

67% of stenoses in this location recurring. Because of the latter finding, a prospective study has recently been commenced comparing PTA with surgical revision for distal stenoses. This should help to define the optimal treatment of these recurrent distal stenoses. Finally, this study highlights the importance of continued surveillance after PTA of a graft stenosis and it is salutary to note that although restenosis occurred at a median of 8.5 months, that seven recurrences occurred more than 12 months after the first PTA.

4 c) THE FATE OF INFRAINGUINAL PTFE GRAFTS AND AN ANALYSIS OF FACTORS AFFECTING THEIR OUTCOME

4 c.i) INTRODUCTION

Whilst autologous saphenous vein is the conduit of choice for infrainguinal bypass surgery (Budd *et al.* 1990), not all patients have suitable vein. When there is no ipsilateral or contralateral long saphenous vein, no short saphenous or arm vein available, the next alternative is prosthetic material, usually polytetrafluoroethylene (PTFE). In addition, some surgeons preferentially use PTFE for above-knee femoropopliteal bypass so that the vein can be saved for more distal reconstruction or for coronary artery bypass grafting (CABG) at a later date (O'Donnell *et al.* 1983). This study looked at a consecutive series of infrainguinal PTFE grafts performed over a 5 year period between January 1988 and March 1993 at the Leicester Royal Infirmary.

4 c.ii) METHODS

All grafts were performed for disabling claudication, rest pain or tissue necrosis. Preoperative arteriography and doppler ultrasonography was performed in all cases. Intraoperative arteriography was used for below-knee procedures to identify a suitable vessel for the distal anastomosis. A distal anastomotic vein collar was used for grafts extending below the knee (Miller *et al.* 1984). Completion arteriography was performed at the end of each operation. Clinical assessment was in an outpatient clinic at 1, 3, 6, 9 and 12 months, and at 6 monthly intervals thereafter.

Graft patency was assessed using the recommendations of the ad hoc committee of the Society for Vascular Surgery (Rutherford 1991). Actuarial patency curves were computed using the Kaplan-Meier method (Kaplan *et al.* 1958) and compared with the Mantel-Haenszel log-rank test (Mantel *et al.* 1959). As in 4 b), multivariate analysis was with a Cox proportional hazard model (Cox 1972). The analysis for this study was performed with the Systat statistical package for the Apple Macintosh.

4 c.iii) RESULTS

There were a total of 257 infrainguinal reconstructions performed at the Leicester Royal Infirmary during the study period, including 95 infrainguinal PTFE grafts in 90 patients. The patient details are shown in Table 4c.1. The PTFE grafts were performed in 65 men and 25 women with a median [range] age of 67 [50-92] years. The operations were carried out for intermittent claudication in 23 cases, rest pain in 37 cases and for tissue necrosis in 35 cases. The graft details are shown in Table 4c.2. Thirty out of the 72 limbs with clinical critical ischaemia fulfilled the European Consensus Document criteria for critical ischaemia (European Working Group on Critical Leg Ischaemia 1991). Sixty-seven reconstructions were to the above-knee popliteal artery and 28 to the below-knee popliteal artery or to a single crural vessel. The primary, primary assisted and secondary patencies of grafts for rest pain or tissue necrosis at 24 months were 37, 42 and 46% respectively with limb salvage and patient survival rates of 65 and 78% respectively. The 30 day mortality in this group was 6%. There was no limb loss in the claudicant group and the perioperative mortality was 5%. The patency curves are shown in Figure 4c.1, whilst the secondary patency, limb salvage and mortality curves for the critically ischaemic limbs are shown in Figure 4c.2.

A univariate analysis of various risk factors was performed to look at their effect on overall graft patency. The risk factors examined were diabetes, smoking, presentation (claudication versus rest pain or tissue necrosis), level of anastomosis (above or below-knee), ankle systolic pressure $\leq$ 50mmHg and number of run-off vessels (1 versus 2 or 3). The actuarial patencies at one year were compared using a log-rank test. The results from this are shown in Table 4c.3. Smoking, an ankle systolic pressure $\leq$ 50mmHg, rest pain or tissue necrosis and single vessel run-off adversely affected graft patency. However, after entering these risk factors into a Cox proportional hazard model, only smoking adversely affected patency ($p<0.01$, Odds ratio 2.8).

Table 4c.1 Patient details.

	Claudication	Rest pain / Tissue necrosis	All
Male	17	48	65 (72%)
Female	3	22	25 (28%)
Median age [range]	69 [52-80]	67 [50-92]	67 [50-92]
Diabetes	4	20	24 (27%)
IHD	10	33	43 (48%)
Hypertension	11	23	34 (38%)
CVD	8	13	21 (23%)
Current smokers	2	25	27 (30%)
Ex-smokers	18	35	53 (59%)
Never smoked	0	10	10 (11%)

IHD = Ischaemic Heart Disease

CVD = Cerebrovascular disease

Table 4c.2 Details of the ischaemic limbs and the site of the bypass grafts.

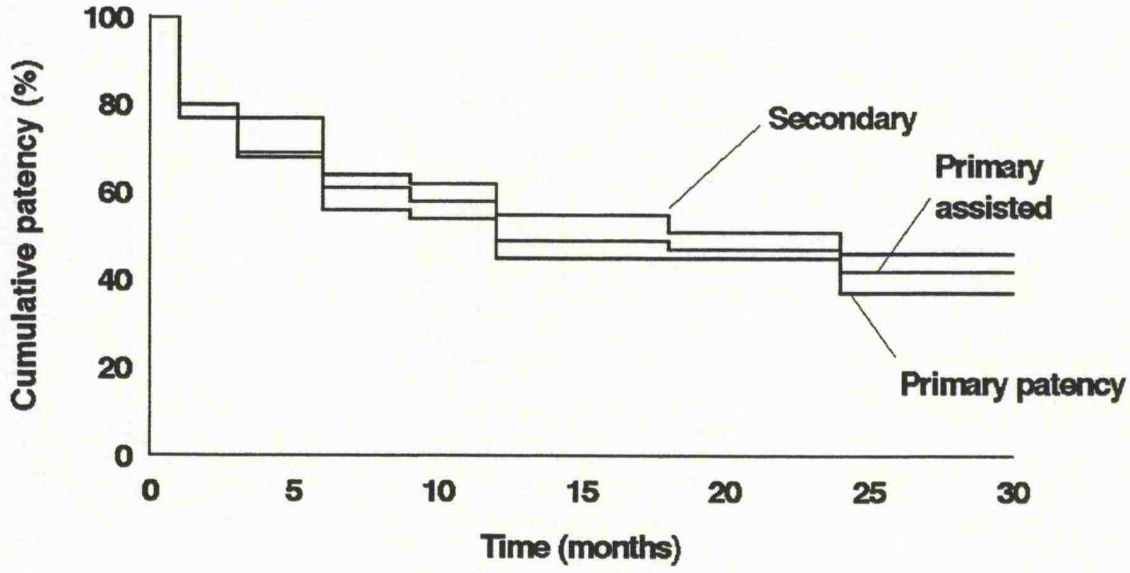
	Claudication	Rest pain / Tissue necrosis	All
Median ASP [Range]	90 [55-140]	60 [0-135]	65 [0-140]
ECD criteria for CLI	0	30	30 (32%)
Run off:			
1 vessel	4	25	29 (31%)
2 vessels	5	27	32 (34%)
3 vessels	14	20	34 (35%)
Proximal anastomosis:			
EIA	0	1	1 (1%)
CFA	20	55	75 (79%)
SFA	0	5	5 (5%)
Other	3	11	14 (15%)
Distal anastomosis:			
AK Popliteal	22	45	67 (71%)
BK Popliteal	1	10	11 (12%)
TP	0	3	3 (3%)
AT	0	7	7 (7%)
PT	0	3	3 (3%)
Peroneal	0	4	4 (4%)

ASP = Ankle Systolic Pressure
ECD = European Consensus Document
CLI = Critical Limb Ischaemia
EIA = External Iliac Artery
CFA = Common Femoral Artery
SFA = Superficial Femoral Artery
AK = Above Knee
BK = Below Knee
TP = Tibioperoneal Trunk
AT = Anterior Tibial artery
PT = Posterior Tibial artery

Table 4c.3 Univariate analysis of factors affecting secondary graft patency during first post-operative year

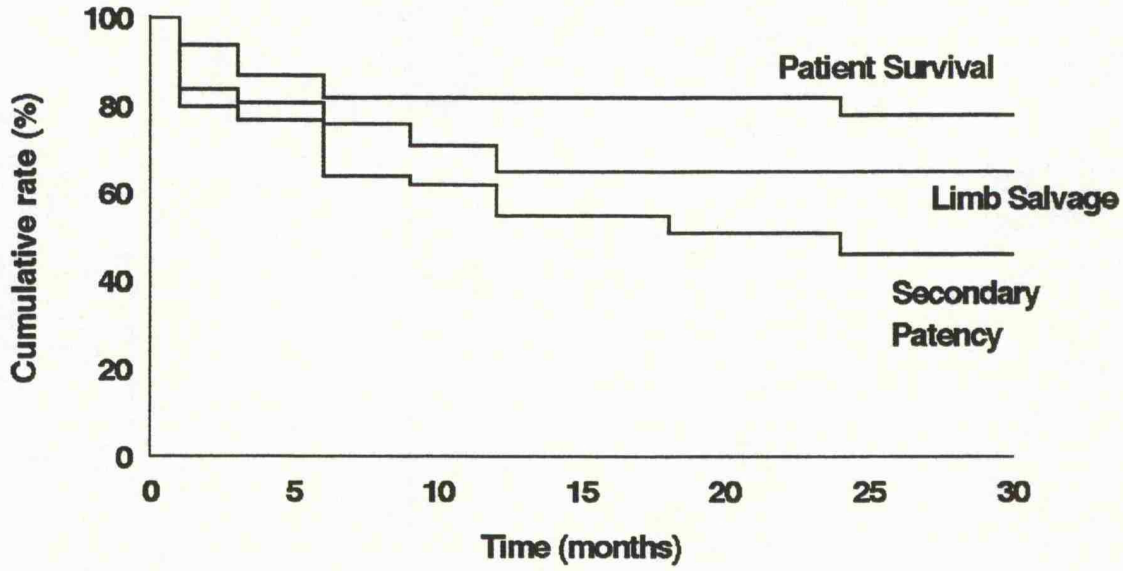
	Secondary graft patency		Log rank
	1 month	12 months	
Diabetic	87	53	p=0.68
Non-diabetic	84	68	
Smoking	70	40	p<0.001
Non-smoking	91	75	
ASP ≤ 50mmHg	73	43	p=0.006
ASP > 50mmHg	90	76	
Presentation			
Claudication	100	91	p=0.001
Rest pain / Tissue necrosis	80	55	
Distal anastomosis			
Above-knee	89	70	p=0.07
Below-knee	74	52	
Run-off			
1 vessel	74	50	p=0.04
2 or 3 vessels	89	70	

Figure 4c.1 Patency curve showing primary, primary assisted and secondary patencies for PTFE grafts.



Primary	72 59	50	35	24	16	11
Primary assisted	72 59	50	36	26	17	11
Secondary	72 61	52	41	28	21	13

Figure 4c.2 Survival curve showing secondary patency, limb salvage and mortality for critically ischaemic PTFE grafts.



Patient Survival	70 65	62	51	42	33	25
Limb Salvage	72 62	55	44	33	26	18
Secondary	72 61	52	41	28	21	13

4 c.iv) DISCUSSION

Whilst autologous vein is the conduit of choice for arterial bypass surgery, PTFE is an acceptable alternative for above-knee popliteal surgery (Sterpetti *et al.* 1985, McCollum *et al.* 1991, O'Riordain *et al.* 1992, Killewich *et al.* 1990), at least in the short term. Some surgeons justify the use of PTFE preferentially for above-knee bypasses so that the vein can be spared for later distal surgery (Quinones-Baldrich *et al.* 1988a) or for a subsequent CABG. However, the perceived need to 'spare' the vein does not appear to be real in a number of studies (Sterpetti *et al.* 1985, Houser *et al.* 1984). A review of the available literature on the patencies of PTFE and synthetic grafts by Michaels (Michaels 1989) came to the conclusion that

autologous vein is the conduit of choice at all levels of bypass and that vein preservation for later use was rarely required.

During the period of this study, PTFE grafts were used preferentially in the above-knee position when a bypass graft was required. However, during the same period, there has been an increasing use of PTA as the first line treatment for isolated femoropopliteal occlusions (Sayers *et al.* 1993c). A distal anastomotic vein collar was used for infragenicular reconstructions when PTFE was the only available conduit (Wolfe *et al.* 1991).

The preoperative risk factors of rest pain or tissue necrosis, an ankle systolic pressure ≤ 50mmHg, a single vessel run-off and smoking all adversely affected patency in a univariate analysis. These risk factors have been shown to affect patency of PTFE infrainguinal bypass grafts in other studies (Rutherford *et al.* 1988, Prendiville *et al.* 1990, McCollum *et al.* 1991). However, in a multivariate analysis, the only one of these inter-related variables which was significant in this study was smoking. These results support the argument that infragenicular bypass with PTFE is justified providing the patient stops smoking.

There was no difference in this retrospective series between the primary and primary assisted patencies of the PTFE grafts. This contrasts with vein grafts where the primary assisted patency is superior to the primary patency, a reflection of the intervention for vein graft stenoses detected by graft surveillance. At the time of this study, prosthetic grafts were not routinely followed up in a surveillance programme. A surveillance programme was subsequently commenced to assess the impact of surveillance on synthetic graft patency.

4 d) THE EFFECT OF A SURVEILLANCE PROGRAMME ON THE PATENCY OF SYNTHETIC INFRAINGUINAL BYPASS GRAFTS

4 d.i) INTRODUCTION

Whilst vein graft surveillance is widely acknowledged to be of benefit in improving graft patency at least in the first year after arterial bypass surgery, the need for a surveillance programme for synthetic infrainguinal bypass grafts has not been demonstrated. In order to ascertain whether a surveillance programme could improve the overall patency of synthetic grafts, a prospective study of synthetic graft surveillance was undertaken.

The reported patency rates for synthetic materials vary considerably with figures of 40-90% being quoted at 1 year(Williams *et al.* 1985, Charlesworth *et al.* 1985, Taylor *et al.* 1987, Prendiville *et al.* 1990, O'Riordain *et al.* 1992), and values of 18-60%(Williams *et al.* 1985, Charlesworth *et al.* 1985, Taylor *et al.* 1987, Budd *et al.* 1990, Prendiville *et al.* 1990, O'Riordain *et al.* 1992) at 5 years. These figures are inferior to the reported patency values for vein grafts. The most common reasons for failure of synthetic grafts are progression of atherosclerotic disease(Veith *et al.* 1980, O'Donnell *et al.* 1984, Sterpetti *et al.* 1985, Taylor *et al.* 1987, Quinones-Baldrich *et al.* 1991) and anastomotic intimal hyperplasia (Clowes *et al.* 1986). This contrasts with vein grafts where intrinsic stenoses due to intimal hyperplasia represent the commonest cause of graft failure between 1 month and 1 year after surgery (McNamara *et al.* 1967, Whitney *et al.* 1976, Berkowitz *et al.* 1992, Mills *et al.* 1993). Few centres enter prosthetic grafts into surveillance programmes as the benefits of surveillance for these grafts has yet to be proven.

4 d.ii) METHODS

All patients undergoing an infrainguinal bypass procedure between January 1st 1992 and December 31st 1994 using prosthetic material were prospectively entered into a surveillance programme. Surveillance of these grafts took place at 3 month intervals after the

initial operation, providing the graft was still patent. The results described include those grafts which failed before they could attend the surveillance clinic.

All patients attending the surveillance clinic had a clinical history taken, and had an ABPI measurement followed by a colour-coded duplex scan (Diasonics Spectra, Diasonics Sonotron, Bedford, U.K.) of the graft inflow, outflow and the proximal and distal anastomoses. This took place in the Vascular Studies Unit where the patient was seen at each visit by an experienced technician. A peak systolic velocity ratio of >3.0 at the anastomosis was taken to represent a significant anastomotic stenosis. A segmental peak velocity ratio >2.0 in the native inflow or run-off vessel was also taken as representing a significant stenosis. Initial treatment of anastomotic or native artery stenoses was by Percutaneous Transluminal Angioplasty (PTA).

Figures 4d.1 to 4d.4 show some of the views obtained with a duplex scanner during surveillance of PTFE grafts.

Graft patency was assessed using the recommendations of Rutherford (Rutherford 1991) based on the suggestions of the Ad Hoc Committee on Reporting Standards of the Society for Vascular Surgery (Rutherford *et al.* 1986). Primary patency was defined as uninterrupted patency after the initial surgery with no further procedure performed on the graft. Primary assisted patency requires that the graft remains patent but procedures such as PTA can be performed to prevent subsequent graft failure. Secondary patency allows procedures such as thrombolysis or thrombectomy to be performed to salvage an occluded graft. Patients who died during follow up were censored to their last clinic attendance. Statistical analysis was performed using the statistical package SPSS for Windows (SPSS, Chertsey, U.K.).

Figure 4d.1 Colour duplex scan of a PTFE graft at the proximal anastomosis with the femoral artery. There is some turbulence but no narrowing.

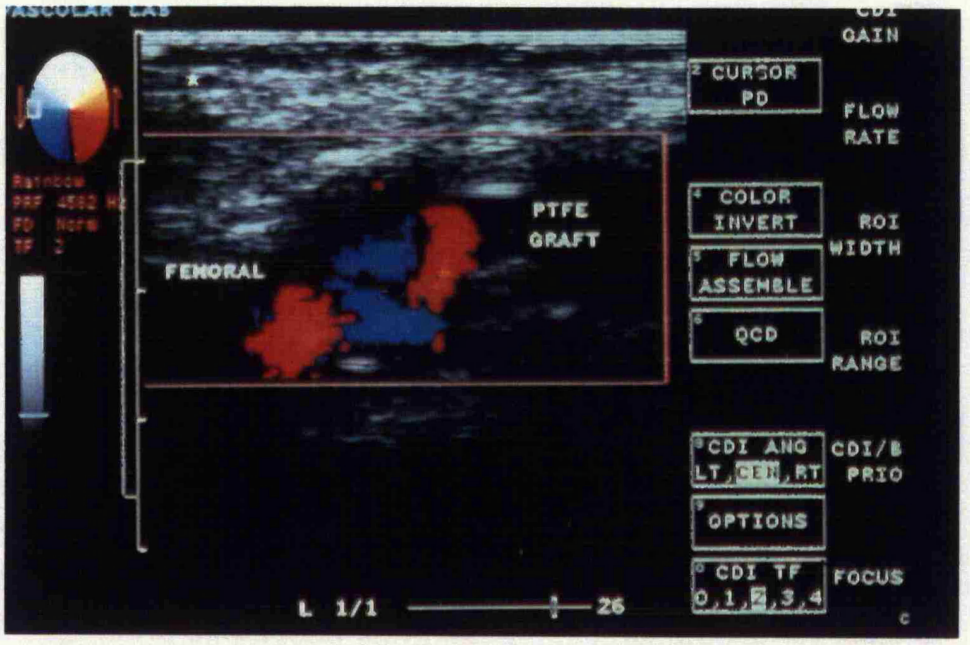


Figure 4d.2 B-mode ultrasound of a distal anastomosis of a PTFE graft with a 'Miller cuff' of vein between the PTFE and the distal artery.

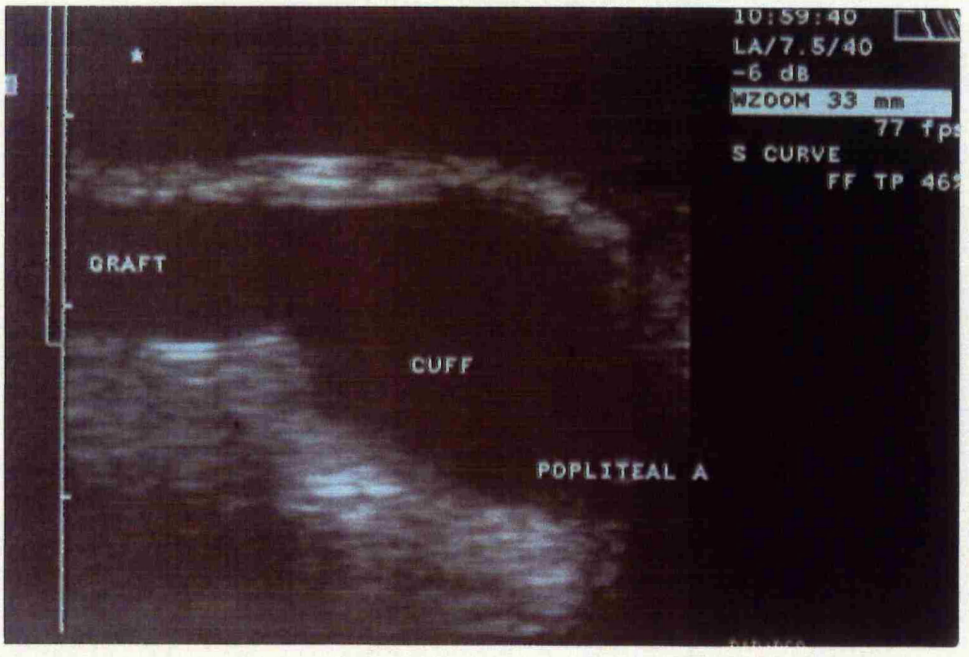


Figure 4d.3 Colour duplex scan of the distal anastomosis shown in Figure 4d.2. There is considerable turbulence at the distal anastomosis.

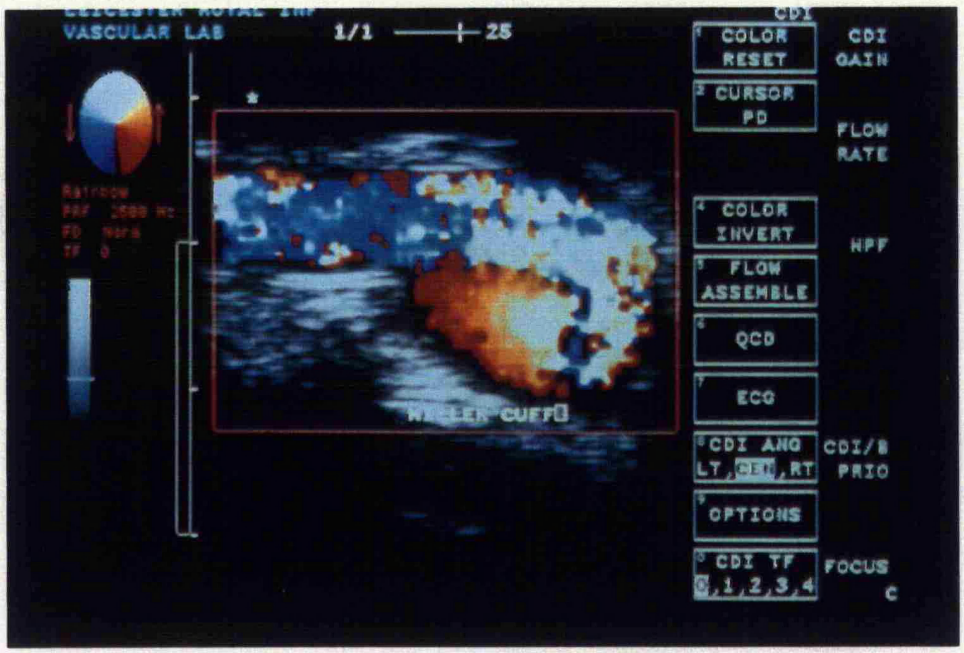
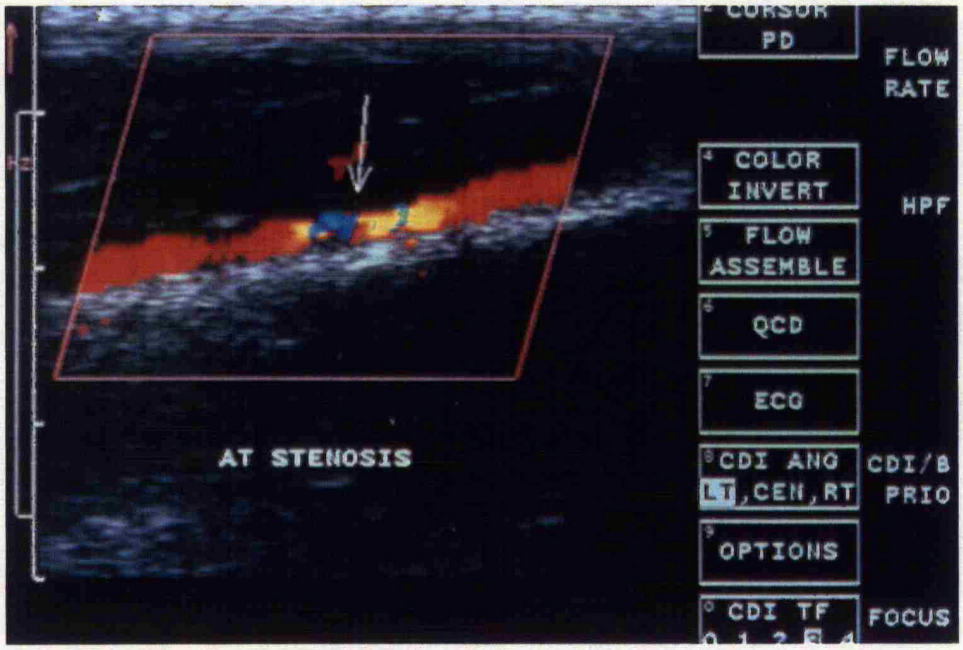


Figure 4d.4 Colour duplex scan of the anterior tibial artery distal to the PTFE graft with a stenosis in the artery. This is amenable to treatment with PTA.



4 d.iii) RESULTS

There were a total of 69 synthetic grafts in 65 patients. The median [range] patient age was 69 [48-92] years. Forty-five of these patients were male and twenty were female. Twenty (29%) of the patients were smokers at the time of their operation, 18 (26%) were diabetic and 28 (41%) were hypertensive. Nineteen of the operations were performed for intermittent claudication, 42 for chronic critical limb ischaemia and 8 for acute lower limb ischaemia.

In total, 49 above-knee popliteal, 6 below-knee popliteal, 12 femorodistal and 2 iliopopliteal grafts were performed using either PTFE (56 grafts), polyurethane ("Corvita") (10 grafts) or tanned bovine vein ("Procol") (3 grafts). The type of graft used at each position is shown in Table 4d.1. A "Miller collar" (Miller *et al.* 1984) of vein was used at the distal anastomosis in 30 (44%) cases. The median [range] follow up was 9 [1-34] months.

Table 4d.1 Graft details.

	Material used			
	PTFE	Corvita	Procol	Miller cuff
Iliopopliteal	2	0	0	0
Above-knee popliteal	38	9	2	16
Below-knee popliteal	6	0	0	3
Femorodistal	10	1	1	11

Sixteen grafts failed within the first 30 days post-operatively giving an overall 30 day patency rate of 77%. Seven patients died perioperatively producing a perioperative mortality rate of 10%. Six of these deaths were in patients having surgery for limb-threatening chronic critical ischaemia, the other patient had surgery for an acutely ischaemic limb. Five of the patients who died in the perioperative period did so after their grafts failed. Fifty-one patients remained alive with a functioning graft to enter the surveillance programme at 1 month.

After the initial 30 day period, 14 grafts occluded, 12 in the interval between surveillance clinic attendances. Five grafts were found to have a treatable lesion by the surveillance programme but 2 of these occluded prior to PTA. A further 2 grafts had treatable lesions detected in the popliteal artery distal to the anastomosis which were treated successfully by PTA.

The 7 grafts with abnormal findings at surveillance are shown in Table 4d.2. The primary, primary assisted and secondary patencies at 12 months were 54%, 55% and 55%. At 24 months the figures were 39%, 46% and 46%. The patency rates are demonstrated in the survival curve in Figure 4d.5. There is little difference between the primary assisted and secondary patency rates.

Whilst there is a preponderance of 'corvita' grafts among those found to have treatable lesions, there was no significant difference between the graft materials in the number of unexpected graft occlusions ($p=0.44$, Log Rank test).

The use of a Miller collar was associated with a decreased incidence of anastomotic stenosis. None of the 30 grafts with a Miller collar developed a stenosis compared to 5 of the 39 grafts without a collar. However, this was not statistically significant ($p=0.09$ Log Rank test). There was an increased incidence of graft occlusion in those grafts with a Miller collar (17 of 30 compared to 13 of 39, $p=0.03$ Log Rank test). The higher rate of graft occlusion in those grafts with a Miller collar reflects the higher failure rate of prosthetic grafts anastomosed onto small tibial vessels. In a Cox multivariate analysis (Cox 1972) of risk factors, there was no significant difference in occlusion ($p=0.53$) or stenosis ($p=0.95$) rates between those grafts with or without a Miller collar. Multivariate analysis also showed no significant effect on graft occlusion or stenosis rate with risk factors such as diabetes mellitus, hypertension, ischaemic heart disease or continued smoking (as admitted by the patient). The operation performed, the

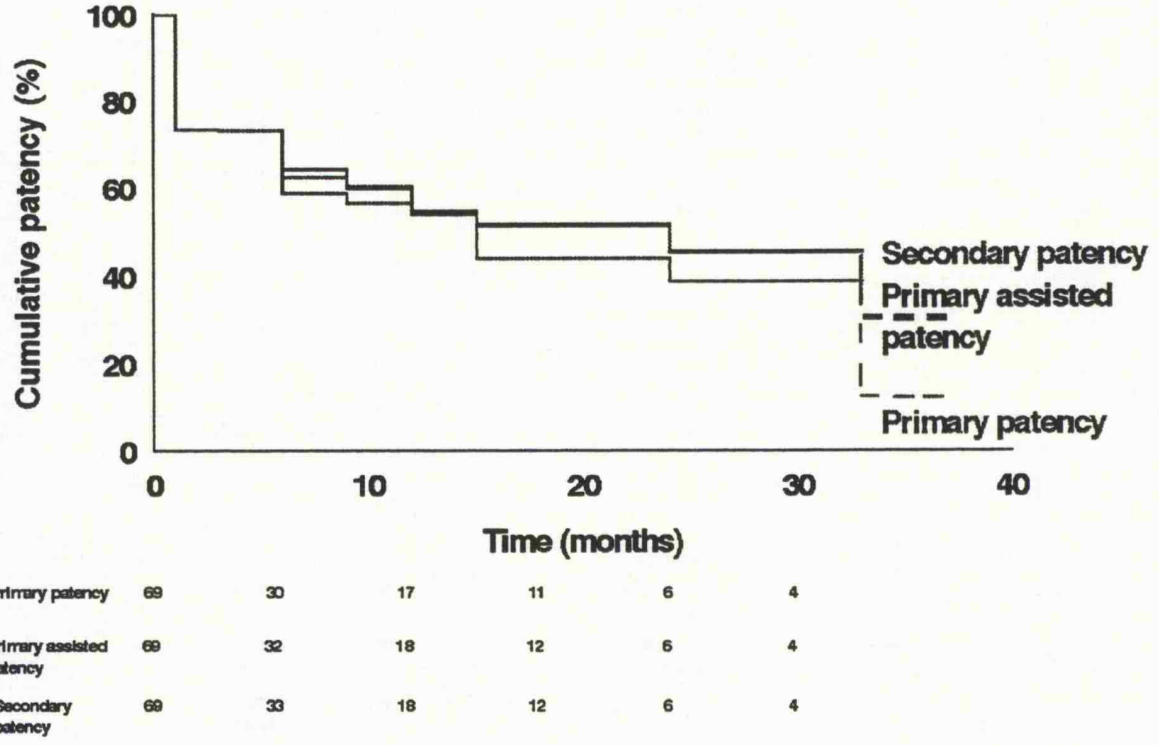
material used and the indication for the operation had no significant effect either, though the numbers of grafts in each group are too small to draw any definite conclusions.

Table 4d.2 Abnormalities detected by surveillance.

Patient	Graft	Details
66yr Male	Corvita AK fempop	2.5x velocity increase in distal popliteal artery at 22 months post-op. Had PTA at 25 months.
78yr Female	Corvita AK fempop	4x velocity increase in popliteal artery at 12 months post-op. Had PTA at 14 months.
63yr Male	Corvita AK fempop	3x velocity increase at distal anastomosis at 5 months post-op. Angiogram at 9 months post-op showed occluded graft.
79yr Male	PTFE AK fempop	2.5x velocity increase at distal anastomosis at 6 months post-op. Angiogram at 7 months post-op showed occluded graft.
66yr Female	Corvita AK fempop.	2.5x velocity increase at distal anastomosis at 3 months post-op. Had successful PTA at 5 months.
65yr Female	PTFE AK fempop	2.5x velocity increase at 29 months post-op. Had PTA of distal anastomosis and popliteal artery at 30 months.
72yr Female	Corvita AK fempop	3x velocity increase at distal anastomosis and 2x velocity increase inflow at 12 months post-op. Had PTA at 12 months.

AK = Above Knee

Figure 4d.5 Patency curves showing primary, primary assisted and secondary patencies. The numbers at risk are shown at the bottom. A broken line is used where the standard error is greater than 10%.



Primary patency	69	30	17	11	6	4
Primary assisted patency	69	32	18	12	6	4
Secondary patency	69	33	18	12	6	4

4 d.iv) DISCUSSION

Graft surveillance for vein grafts is of proven benefit in maintaining graft patency (Berkowitz *et al.* 1981, Grigg *et al.* 1988a, Disselhoff *et al.* 1989, Moody *et al.* 1990, Mills *et al.* 1990, Brennan *et al.* 1991, Berkowitz *et al.* 1992, Harris 1992, Idu *et al.* 1992, London *et al.* 1993, Mills *et al.* 1993, Sayers *et al.* 1993b, Lundell *et al.* 1995). However, the benefits from surveillance of PTFE grafts are not so clear. The main causes of PTFE graft failure are progression of atherosclerosis and anastomotic intimal hyperplasia and any surveillance programme would have to detect and allow treatment of these problems before graft occlusion occurred (Sanchez *et al.* 1993a). As the results of treating a failed graft are worse than treating a failing graft, a surveillance programme would be of benefit in improving graft patency if it

was cost effective and detected enough treatable lesions prior to graft occlusion (Sanchez *et al.* 1993b). Stenoses can be treated by open operation or by PTA. Inflow and run-off disease can also be treated by PTA or by further arterial bypass surgery. If the graft has already occluded, then it may be treated by thrombolysis or thrombectomy prior to treating the underlying cause for the occlusion (Veith *et al.* 1980) but the results are not as good.

There are few reports in the literature of surveillance programmes of synthetic grafts. Sanchez et al. retrospectively reviewed the results of their treatment of 91 'failing' PTFE grafts performed over a 12 year period (Sanchez *et al.* 1993b). These were grafts found to have a problem during routine follow up. They found 43 inflow and 83 outflow lesions in addition to 10 lesions within the graft itself and 8 anastomotic lesions. These grafts had all been followed up in an outpatient setting at 1-2 monthly intervals for the first year, 3 monthly intervals for the second year and at 3-6 monthly intervals thereafter. Duplex scanning was available during the last 6 years of their study and 'most' patients were said to have had a duplex scan during this time. However, the study is retrospective and the follow up protocol was not standardised. In addition, no mention is made of the number of unexpected graft failures during this time so there is some uncertainty as to how successful their programme was at picking up problems. They conclude that a surveillance programme *may* allow detection and treatment of graft-threatening lesions but that a prospective study is needed.

Lalak et al. reported a prospective surveillance programme of 69 PTFE grafts over a 4 year period (Lalak *et al.* 1994). Their report included only those patients who left hospital with a functioning graft and patients were seen at 1 month, 3 months then at 6 monthly intervals. There were no inflow or runoff problems found in this series but there were 3 proximal and one distal anastomotic stenoses requiring treatment. Twenty-seven grafts occluded during the 3 year follow up despite having been seen in the surveillance clinic during the preceding 6 months. They concluded that surveillance was of little benefit in improving overall synthetic graft patency.

A prospective randomised study of graft surveillance by Lundell et al. (Lundell *et al.* 1995) included 43 PTFE or composite grafts. Twenty-three of these grafts were allocated to

intensive surveillance with ABPI measurements and duplex scans at 1 month, 3 months then 3 monthly intervals. The remaining 23 grafts underwent 'routine surveillance' at 1month then 12 monthly intervals. These grafts had ABPI measurements but did not have a duplex scan. Two of their PTFE grafts in the intensive surveillance group developed anastomotic steroses that were treated and one other PTFE graft with a stenosis occluded before it could be treated. Ten of their PTFE grafts and 3 of their composite grafts in the intensive surveillance group occluded without warning. There was no statistical difference between intensive and routine surveillance at one year but the numbers of PTFE grafts in the study were too small to make statistical comments beyond this time.

Before deciding whether or not a screening programme is useful, an assessment of the effectiveness and the expense of the screening method is required. In this study, haemodynamic abnormalities were detected in 5 grafts and in 2 run-off vessels which were believed to be indicative of threatening graft occlusion. Two of the grafts with anastomotic stenoses occluded before the patient returned to have a remedial procedure. Whilst surveillance was certainly useful in these 7 patients, 12 grafts failed unexpectedly during follow up. Haemodynamic abnormalities were not detected despite the patients being in the surveillance programme. Assuming the duplex scanning was carried out effectively, and we believe it was, the conclusion must be that surveillance at 3 monthly intervals is of limited use in improving the patency of synthetic infrainguinal bypass grafts.

There are several possibilities why the grafts occluded between surveillance appointments and why the overall graft patency was not substantially altered. Synthetic grafts are more thrombogenic than vein grafts and small haemodynamically insignificant stenoses causing minimal flow disturbance, which would not jeopardise vein graft patency may lead to occlusion of synthetic grafts. Alternatively, the intimal hyperplasia at the anastomoses of synthetic grafts may progress more rapidly than that in vein grafts. Thus an insignificant stenosis seen on duplex may advance to cause a graft occlusion within the 3 month interval between surveillance appointments. The surveillance interval in Lalak's study was even longer - 6 monthly after the first 3 months and there was a higher rate of unexpected graft occlusion. It would appear that the detection of treatable lesions in synthetic grafts before occlusion occurs

is fraught with difficulties. A surveillance programme with a shorter interval between visits, perhaps monthly, may detect more treatable lesions but the costs of the programme would rise considerably. A prosthetic graft surveillance programme, as described above, cannot therefore be justified.

Chapter 5 - The effect of recombinant growth factors on cultured human saphenous vein smooth muscle cells

5 a) INTRODUCTION

5 b) METHODS OF CELL CULTURE

5 c) COMPARISON OF RECOMBINANT GROWTH FACTORS

i) INTRODUCTION

ii) METHODS

iii) RESULTS

5 d) EFFECT OF INCREASING GROWTH FACTOR CONCENTRATION

i) INTRODUCTION

ii) METHODS

iii) RESULTS

5 e) COMPARISON OF THE 2 ISOMERS OF PDGF

i) INTRODUCTION

ii) METHODS

iii) RESULTS

5 f) SUMMARY OF RESULTS

5 g) CONCLUSIONS

5 a) INTRODUCTION

As discussed in Chapter 2, there are numerous growth factors which have been shown in animal or in culture experiments to have a proliferative effect on vascular SMCs. However, there are variations between species and between SMCs derived from the arterial and the venous systems. As human saphenous vein is the conduit most frequently used in arterial bypass surgery, the ideal cell to study is the human saphenous vein smooth muscle cell.

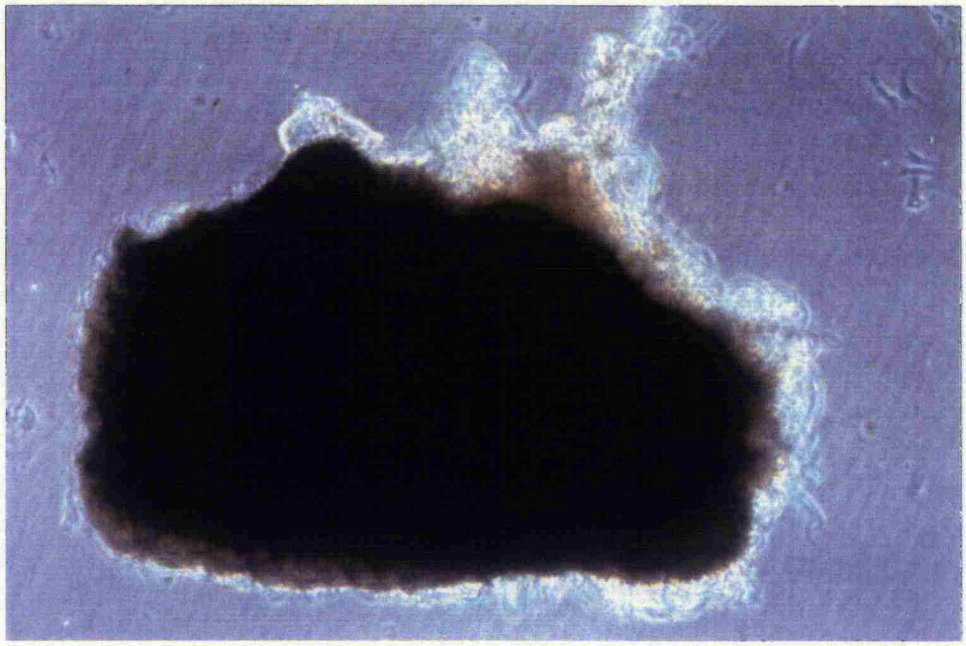
Previous work from our department has shown that a paracrine mediator of SMC proliferation is produced in the human saphenous vein organ culture model (Allen *et al.* 1994). This mediator was able to promote intimal hyperplasia in a denuded segment of vein in a coculture model. The mediator is produced by the intact segment of vein and is likely to be a product of the intact endothelium. The mediator was able to stimulate proliferation of human saphenous vein SMCs in the denuded segment of vein when culture medium supplemented with 30% FCS was not. The mediator or mediators of this proliferation are likely to be one of a number of growth factors. As a way of studying which growth factors have a significant proliferative effect on human saphenous vein SMCs, various recombinant cytokines which are known to be present in either venous endothelium or venous SMCs were added to human saphenous vein SMCs in culture.

5 b) METHODS OF CELL CULTURE.

Human saphenous vein SMCs were obtained using an explant technique based on the method described by Chamley-Campbell et al. (Chamley-Campbell *et al.* 1979). Ethical committee approval was obtained to use segments of saphenous vein excess to surgical requirements obtained at aortocoronary or peripheral arterial bypass surgery. Samples were obtained from theatre and transported in a calcium-free Kreb's solution at 4°C to the laboratory. In the laboratory, the samples were placed in a sterile petri dish within a laminar flow hood to maintain sterility. Then, using small dissecting forceps and scissors, the segment of vein was dissected free of any fat, excess adventitial tissue or suture material. The vein was then opened up along its longitudinal axis. The endothelium was removed by a scraping action

over the luminal surface with a sharp scalpel blade. The vein was then gently washed with minimal essential medium (MEM) and placed in 1.8mls of "smooth muscle cell culture medium". This medium consisted of RPMI 1640, supplemented with L-Glutamine 2mmol/l, penicillin 50U/ml and streptomycin 50μg/ml and 15% FCS. The vein was then chopped into small fragments (~ $1mm^3$) within the medium. The vein fragments with their medium were then transferred to a T25 culture flask and placed in a tissue culture incubator at 37°C with a maintained humidified atmosphere containing 95% air and 5% CO_2 to maintain the pH of the medium at a constant level. The 1.8mls of medium was just enough to cover the surface of the flask but was not enough to allow the vein explants to float (Figure 5b.1). The medium was changed only when a colour change (from red to yellow, indicating a fall in pH) could be seen. This was usually at 1-2 weeks.

Figure 5b.1 Human saphenous vein medial explants in culture



After 2-4 weeks in culture, SMCs could be seen to be growing out from the explants (see Figure 3b.1). Once this had occurred, the medium was changed every 2-3 days. Only half the medium (0.9mls) was replaced each time. When the cells had become subconfluent, they were subcultured by removing the SMC medium, washing with MEM, then adding 0.1%

Trypsin + 0.02% EDTA (T/E). Two mls of T/E were added, but 1ml was removed almost immediately after washing over the cells in the flask. The flask was then placed back in the incubator for between 2-5 minutes until the SMCs were seen to have detached from the surface of the flask. The trypsin was then neutralised with SMC medium containing 15% FCS, the cells were centrifuged at 300g for 7 minutes in a CR422 centrifuge (Jouan, St. Nazaire, France) and resuspended in 15 mls SMC medium and plated out in 3 sterile T25 flasks. These cells could then be grown to confluence and repassaged as necessary using the same methods used for the initial subculturing. A 1:3 ratio was used for subculturing each time. All cells were used between passages 2 and 5. There has been some suggestion that further passaging leads to a loss of viability (Chamley-Campbell *et al.* 1979).

Once enough cells were available for an experiment they were rendered quiescent by replacing their medium with "growth arrest medium". This was essentially the same medium with RPMI, L-glutamine, penicillin and streptomycin but with only 0.4% FCS. This level of serum is enough to stop the cells from dying off but does not stimulate proliferation. Other investigators have used levels of 0.4% (Ikeda *et al.* 1990, Chan *et al.* 1993), 0.5% (Hansson *et al.* 1988, Winkles *et al.* 1991, Kanthou *et al.* 1992, Gilbertson *et al.* 1992) or 1% (Raines *et al.* 1989, Schollmann *et al.* 1992) serum to growth arrest SMCs. The cells were growth arrested for a period of 72 hours to ensure that they were all at the G_0 stage in the growth cycle before the proliferation assays were begun. After the 72 hour period of growth arrest, the cells were harvested using T/E. This was neutralised with MEM supplemented with 5% FCS and the cells were then immediately centrifuged and resuspended in plain MEM. The number of cells present in the MEM was calculated using a haemocytometer and the cells were again centrifuged and resuspended this time at a known concentration of 10^4 cells/ml in either 2.5% FCS medium or 15% FCS medium. The cells were plated out in 24 well plates so that there were 10^4 cells in each well. A separate plate was used for the study of each growth factor. Each plate contained 10^4 cells in 21 of the 24 1ml wells. One plate served as a control, containing only RPMI with L-glutamine, penicillin and streptomycin supplemented with 2.5% FCS, one acted as a positive control with the standard 15% FCS medium, and the remainder contained the 2.5% FCS medium with one of the growth factors under investigation. The value of 2.5% FCS was chosen, as this level of serum was found to be adequate to allow the SMCs

to have a slow turnover rate whilst not completely arresting the cells. At lower levels of serum, the cells could not be stimulated to proliferate following the addition of various growth factors. Other investigators have shown incorporation of thymidine into SMCs at lower serum levels (Hwang *et al.* 1992, Panetta *et al.* 1992a) but cell replication is inhibited without a minimum amount of serum present. Plasma-derived serum (2%) (Libby *et al.* 1988b) or between 0.5-3% FCS have been used to provide "minimal stimulation" by other workers (Schollmann *et al.* 1992, Nugent *et al.* 1993, Kimura *et al.* 1992).

5 c) COMPARISON OF RECOMBINANT GROWTH FACTORS.

5 c.i) INTRODUCTION

Various growth factors have been shown to stimulate SMC proliferation in different animal models. The growth factors investigated in this experiment were those considered by Ross to have an effect on the SMC proliferation in atherosclerosis in humans and were known to be produced by vascular endothelial or smooth muscle cells (Ross 1993). These were platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), transforming growth factor beta (TGF-β), tumour necrosis factor alpha (TNF-α), interleukin-1α (IL-1α) and insulin-like growth factor-1 (IGF-1).

5 c.ii) METHODS

Platelet-derived growth factor-BB (PDGF-BB) was obtained from Sigma (Sigma Chemical Co. Ltd., Poole, Dorset). This was a recombinant human growth factor expressed in E. coli. The -BB isomer of PDGF was used as this is the form predominantly produced by endothelial cells. It is also able to stimulate both PDGF-A and -B receptors (Hart *et al.* 1988) and is therefore likely to have a greater proliferative effect than PDGF-AA or PDGF-AB (D'Amore *et al.* 1993). The growth factor was supplied as a lyophilised powder which was reconstituted in a 4mM hydrochloric acid solution containing 1mg/ml of bovine serum albumin (BSA). The resulting solution was sterilised by passage through a 0.22μm filter pre-blocked with BSA. Aliquots were frozen and stored at -20°C for up to 3 months. The concentration

used in this experiment was 10ng/ml. This concentration produces the maximal proliferative effect in tissue culture experiments (Miyazono *et al.* 1989, Sachinidis *et al.* 1990, Yamamoto *et al.* 1994) and was at the upper range recommended by the manufacturer for cell culture work.

Basic fibroblast growth factor (bFGF) was also obtained from Sigma. This was a recombinant human product expressed in E. coli. It was supplied as a sterile lyophilised powder. It was reconstituted in tissue culture medium containing 2.5% FCS. Aliquots were frozen and stored at -20°C. The growth factor was used at a working concentration of 10ng/ml, which was at the upper end of the range recommended for tissue and cell culture work by the manufacturer. Some workers have suggested a maximal proliferative effect at concentrations between 1-5ng/ml (Klagsbrun *et al.* 1989, Nugent *et al.* 1993).

Transforming growth factor beta-1 (TGF-β1) was obtained from Sigma. It was derived from human platelets and supplied as an aseptically lyophilised product containing 1μg of TGF-β1 and 50μg of BSA. It was reconstituted using a 4mM hydrochloric acid solution containing 1mg/ml of BSA. Aliquots were frozen and stored at -20°C. Transforming growth factor beta-1 was the isomer used as this is the most abundant form of TGF-β found in human tissue. It was used at a concentration of 1ng/ml which is at the upper end of its recommended dose for tissue and cell culture work. This dose is equivalent to 40pM which was found to be maximally stimulative in rat aortic SMCs (Stouffer *et al.* 1994).

Tumour necrosis factor alpha (TNF-α) was obtained from Sigma. This is a recombinant human product expressed in yeast. It was supplied in 1ml of sterile frozen phosphate-buffered saline (PBS) solution containing 0.1% BSA. This was diluted as necessary in sterile tissue culture medium containing 2.5% FCS. Aliquots were frozen and stored at -20°C. The growth factor was used at a concentration of 10ng/ml which is at the upper end of the manufacturer's recommended dose for tissue culture.

Interleukin-1α (IL-1α) was obtained from Sigma. This is a recombinant human product expressed in E. coli. It was supplied in 1ml of sterile PBS solution containing 0.1% BSA. This was further diluted as necessary in tissue culture medium containing 2.5% FCS and was frozen

and stored in aliquots at -20°C. The growth factor was used at a working concentration of 25iu/ml, a concentration which produced a maximal effect on SMC proliferation in cell culture work (Ikeda *et al.*1990).

Insulin-like growth factor-1 (IGF-1) was obtained from Calbiochem (Calbiochem-Novabiochem, Nottingham, U.K.). This is a human recombinant product expressed in E. coli. It was supplied as a lyophilised powder which was reconstituted in a 10mM sodium acetate buffer. As the growth factor was not guaranteed to be sterile, this was then filter sterilised using a 0.2μm filter. The growth factor was used at a working concentration of 10ng/ml which is at the upper end of the manufacturer's recommended dose for cell and tissue culture work.

Each of these growth factors was added to 21 of the 1ml wells containing 10^4 SMCs. In addition, there was one plate of wells which contained only medium with 2.5% FCS and no added growth factor, and one plate of wells which contained medium supplemented with 15% FCS but no added growth factor. The experiment was carried out over a 14 day period. The medium was completely replaced in all but 3 of the wells on every 2nd day and new growth factor was added with the new medium. In the remaining 3 wells, the cells were harvested and counted. Harvesting was achieved by removing the tissue culture medium, washing the cells with MEM, and adding 200μl of T/E. The 24 well plate was then placed back in the incubator for 10-20 minutes until the SMCs had detached from the base of the plate. The cell suspension in each well was then aspirated and placed in a 1ml eppendorf. The well was washed out with 800μl of MEM containing 5% FCS which was also added to the eppendorf to neutralise the trypsin. The eppendorfs were then centrifuged in a microcentaur centrifuge (MSE, U.K.) for 5 minutes at 13,000 RPM. This was sufficient to ensure that all the SMCs were pelleted to the bottom of the eppendorf. Then 900μl of the medium was carefully aspirated and was replaced with 100μl of 0.2% trypan blue dye. This mixture of dye, SMCs and medium was then shaken using a 'Vortex-Genie' (Scientific Industries Inc, New York, U.S.A.) and a 100μl aliquot was placed in a haemocytometer to assess the initial concentration of SMCs in the well. The concentration of SMCs in 3 separate wells was measured for each growth factor at each time point and a mean value was calculated for that time point. From this a growth curve could be calculated for each growth factor. A total of 10 experiments were performed using 10 different

clones of SMCs. As there was considerable variation between clones, each curve within one experiment was compared to the control growth curve within the experiment containing only the 2.5% FCS with no added growth factor.

Cell counts were made on every second day and a growth curve was calculated for each growth factor and for the 2.5% FCS control and the 15% FCS positive control. The area under the curve was taken as representing the amount of "growth" over the time period. The areas were calculated using Simpson's rule (see appendix 1). The growth areas were compared to the area for the 2.5% FCS area for each cell clone. This gave a "growth ratio" for each growth factor and for the 15% FCS medium for each SMC clone. The areas were compared using a non-parametric statistical method, a Kruskal-Wallis one way analysis of variance followed by a Wilcoxon matched pairs signed ranks test. The statistics were calculated using the package SPSS for Windows (SPSS, Chertsey, U.K.).

5 c.iii) RESULTS

The Figures below show some of the variability between different clones of SMCs. Figure 5c.1 shows the median growth curves for 2.5% FCS, 15% FCS and for all 6 growth factors. From a straightforward observation of this curve, PDGF and bFGF seem to have a significant proliferative effect on HSVSMC proliferation.

Figure 5c.2 shows the growth ratios for each of the growth factors. The median and 95% confidence interval values are marked by horizontal lines. The broken line represents the ratio "1.0", i.e. the growth ratio for 2.5% FCS.

Table 5c.1 below shows the median growth ratios and the p value for each of the growth factors tested.

Table 5c.1 Growth ratios of SMCs.

Medium / Growth factor	Median growth ratio	p value
2.5% FCS only	1.0	-
15% FCS only	2.6	**0.005**
2.5% FCS + PDGF-BB	2.3	**0.005**
2.5% FCS + bFGF	1.5	**0.007**
2.5% FCS + TGF-β1	1.1	0.2
2.5% FCS + TNF-α	1.1	0.06
2.5% FCS + IL-1α	1.1	**0.04**
2.5% FCS + IGF-1	1.1	0.24

Clearly from the results above, it can be seen that PDGF, bFGF and IL-1α have a significant proliferative effect on HSVSMCs compared to culture medium with 2.5% FCS alone. The proliferative effect of PDGF is very close to that achieved with 15% FCS medium. Basic FGF has a significant effect on SMC proliferation, though it was not as marked in this experiment as that achieved by PDGF. Whilst none of the other growth factors tested had as great an effect on SMC proliferation, the slight increase produced by IL-1α was significant ($p=0.04$). Interleukin-1 exerts its effect on SMC proliferation by inducing the autocrine production of PDGF-AA (Raines *et al.* 1989, Ikeda *et al.* 1990), and this is almost certainly the reason that SMCs in this experiment did respond to IL-1α but not to several of the other growth factors.

Figure 5c.1 Median growth curves for the minimal growth medium (2.5% FCS), maximal growth medium (15% FCS) and the growth factors tested. Each point represents the median of 10 values.

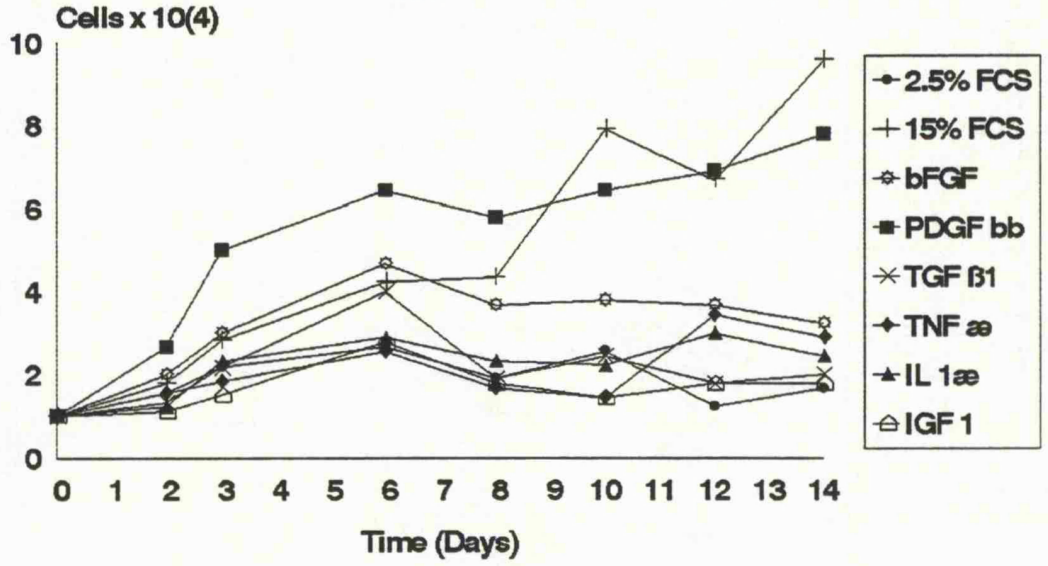
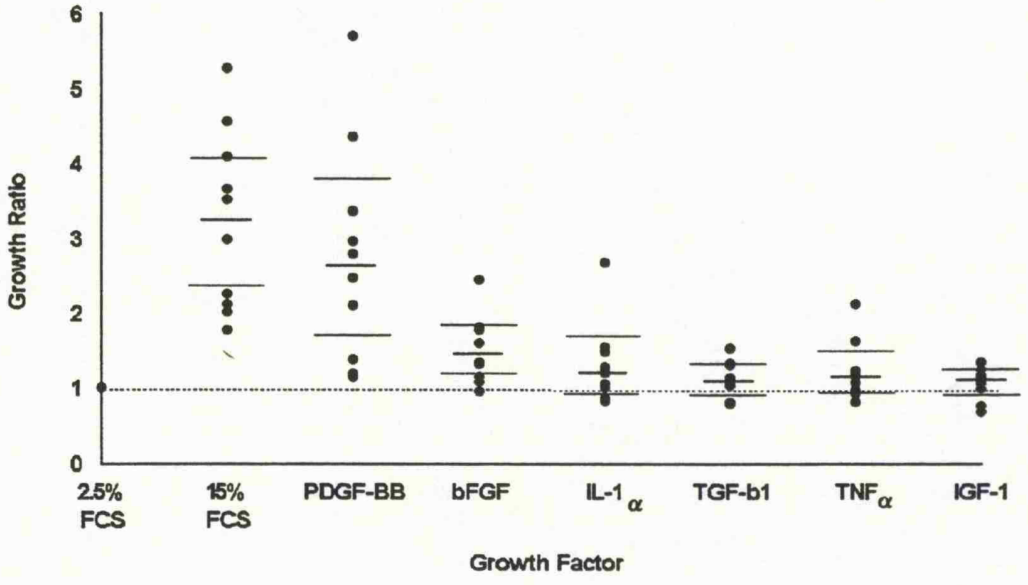


Figure 5c.2 The growth ratios for each of the growth factors and for the 15% medium. The growth ratio is a ratio of the area of the growth curve for each growth factor compared to the area of the growth curve for the minimal growth medium (2.5% FCS). The horizontal lines represent the median values and the 95% confidence intervals.



5 d) EFFECT OF INCREASING GROWTH FACTOR CONCENTRATION.

5 d.i) INTRODUCTION

Whilst all the growth factors were used in this experiment at their recommended maximal levels for tissue culture work, it is possible that using higher doses may have achieved a proliferative effect when the dose used in this experiment did not. Thus 2 growth curves were calculated using twice the concentration of growth factor used in this experiment to see if there was any appreciable difference in cell proliferation.

5d.ii) METHODS

The experiment was conducted as before using different SMC clones for each experiment. Platelet-derived growth factor-BB was used at the same concentration of 10ng/ml but all the other growth factors were used at twice their previous concentrations.

5 d.iii) RESULTS

As shown in Table 5d.1 below, the growth ratios achieved did not differ substantially from those obtained with the original concentration of growth factor.

Table 5d.1 Growth ratios using higher concentrations of growth factors. The ratios are given for both experiments.

Medium / Growth factor	Growth ratio Exp 1	Growth ratio Exp 2
2.5% FCS	1	1
15% FCS	4.1	3.7
2.5% FCS + PDGF-BB at 10ng/ml	1.4	2.9
2.5% FCS + bFGF at 20ng/ml	1.2	1.4
2.5% FCS + TGF-β1 at 2ng/ml	0.6	1.0
2.5% FCS + TNF-α at 20ng/ml	0.6	1.0
2.5% FCS + IL-1α at 50iu/ml	1.0	1.1
2.5% FCS + IGF-1 at 20ng/ml	0.9	0.9

5 e) COMPARISON OF THE 2 ISOMERS OF PDGF.

5 e.i) INTRODUCTION

As discussed in chapter 2, PDGF may exist as one of 3 different isomers, PDGF-AA, PDGF-AB, or PDGF-BB. There are some differences in the actions of these isomers in some animal and culture models, related to the fact that the A chain of PDGF will only bind to the PDGF A/B receptor whilst the B chain will bind to the A/B receptor or to the B receptor (Hart *et al.* 1988). Both chains are capable of stimulating proliferation in various cell types (Hosang *et al.* 1989b). However, the mode of action of PDGF-AA may differ from that of the other 2 isomers, at least in rat SMCs (Sachinidis *et al.* 1990). Whilst the -AB combination is the most common isomer in human platelets (Ross 1989), the -BB combination is found predominantly in endothelium (Kourembanas *et al.* 1989), and the -AA combination in SMCs (Winkles *et al.* 1991). Thus in the coculture model of human saphenous vein (Allen *et al.* 1994), the intact segment of vein could produce either PDGF-BB in the endothelium or PDGF-AA in the intimal SMC layer.

5 e.ii) METHODS

In order to assess the relative efficacies of the two isomers of PDGF to stimulate proliferation of HSVSMCs, an experiment to compare the 2 growth curves was performed. Human saphenous vein segments were obtained as in the previous experiment and SMCs were cultured and passaged as required. All cells were used between passage 2 and 5. When enough cells were available, they were growth arrested for 72 hours in 0.4% FCS as previously described. They were then plated out at a concentration of 10^4 cells/ml in 24 well plates in either 2.5% FCS medium, 15% FCS medium, 2.5% medium with 10ng/ml of PDGF-AA or 2.5% FCS medium with 10ng/ml of PDGF-BB. Both isomers were obtained from Sigma (Sigma Chemical Co. Ltd., Poole, Dorset). They were recombinant human growth factors expressed in E. coli and supplied as lyophilised powders. They were reconstituted in a 4mM hydrochloric acid solution containing 1mg/ml of bovine serum albumin (BSA). The solutions

were sterilised by passage through a 0.22μm filter pre-blocked with BSA. Aliquots of the sterile growth factor were frozen and stored at -20°C for no longer than 3 months.

The medium was changed every second day and new PDGF-AA or -BB was added as required. Cells were counted from 3 wells in each plate on every second day using a haemocytometer as previously described, and a mean value was calculated for the SMC concentration at each time point. Growth curves could then be calculated for the growth factors. A total of 8 experiments were performed using different SMC clones. The growth curves for each medium were compared using a Wilcoxon matched pairs signed ranks test.

5 e.iii) RESULTS

Both the PDGF-AA and the PDGF-BB showed a significant proliferative effect on SMC growth compared to 2.5% FCS alone ($Z= -2.52$, $p= 0.012$ for both growth factors when comparing both the areas and the ratios). PDGF-BB appeared to have a greater stimulatory effect but this was not statistically significant ($Z= -1.61$, $p= 0.11$ for the 2 growth ratios). The values for the areas under the curves and for the growth ratios are shown in the Table 5e.1 below.

Table 5e.1 Median growth areas and growth ratios for PDGF-AA and PDGF-BB.

Medium / Growth factor	Median area under curve	Median growth ratio
2.5% FCS	23.4	1
15% FCS	55.7	2.9
PDGF-AA	35.8	1.8
PDGF-BB	37.3	2.1

The growth curves and growth ratios are demonstrated in Figures 5e.1 and 5e.2.

Figure 5e.1 The median growth curves for PDGF-AA, PDGF-BB, 2.5% FCS medium and 15% FCS medium.

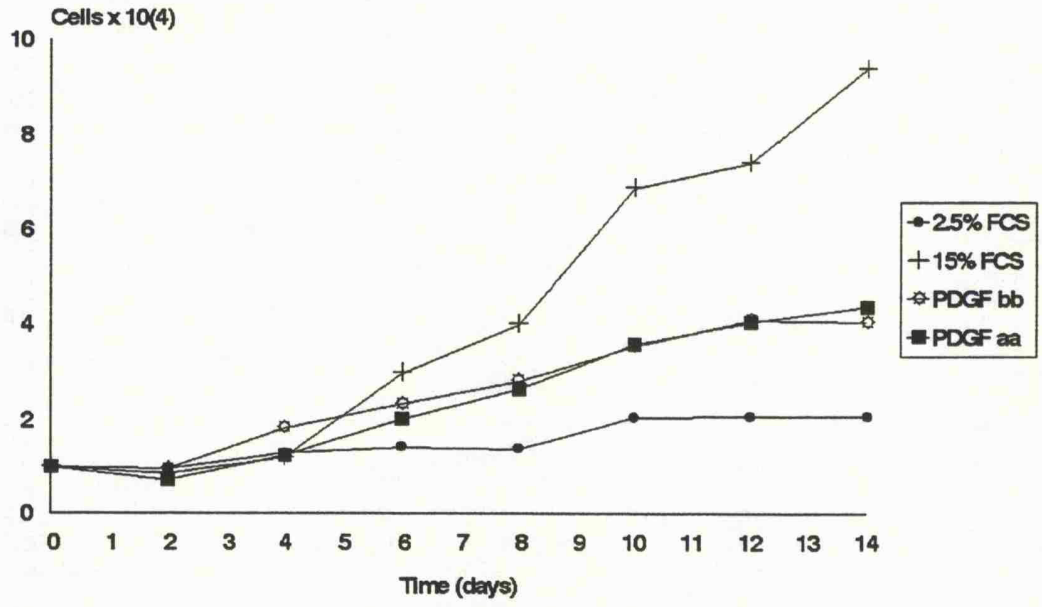
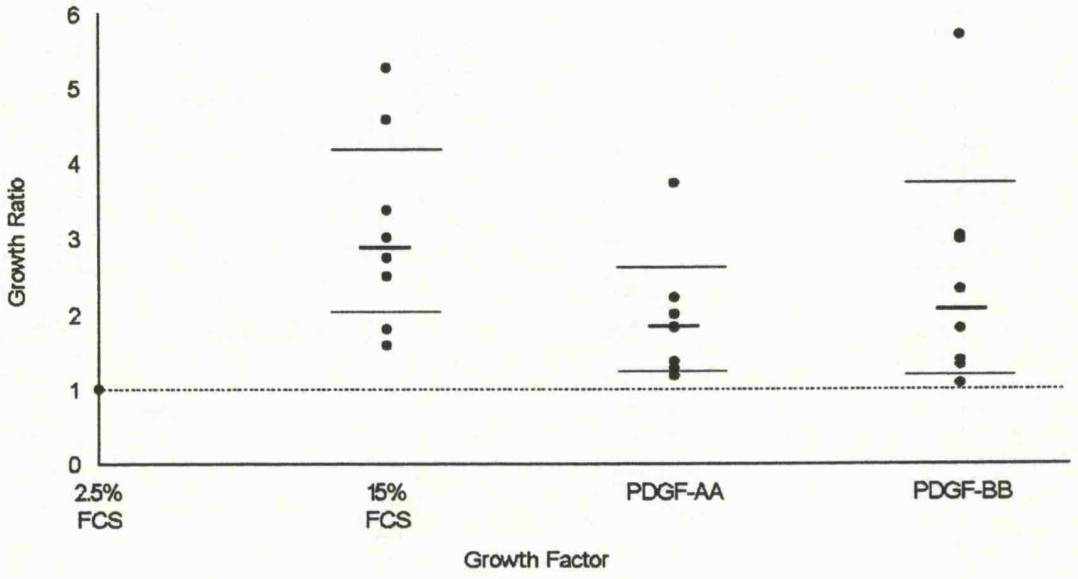


Figure 5e.2 Scatter plot of growth ratios for PDGF-AA, PDGF-BB and 15% FCS. The horizontal lines represent the median values and the 95% confidence intervals.



5 f) SUMMARY OF RESULTS

The above experiments have shown that the proliferation of human saphenous vein SMCs in culture is significantly stimulated by the addition of platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and to a lesser extent interleukin-1α (IL-1α). The median growth ratio for PDGF-BB was 2.3, that for bFGF was 1.5 and that for IL-1α was 1.1. A doubling of the concentration of the growth factors did not appear to increase their ability to stimulate the SMCs to proliferate. Platelet-derived growth factor (both the -BB and -AA isomers) was by far the most stimulatory of the growth factors used. In the experiment in

5 e) there was no significant difference between the -BB and -AA isomers in their ability to stimulate proliferation in the saphenous vein SMCs.

5 g) CONCLUSIONS

In conclusion, human saphenous vein SMCs can be isolated and cultured from segments of human saphenous vein discarded at the time of aortocoronary or peripheral arterial bypass surgery. These SMCs grow well in a culture medium supplemented with 15% foetal calf serum. After a 72 hour period of growth arrest, these cells will grow quite rapidly again if placed in the same medium containing 15% FCS (the positive control medium). If placed in the same medium containing only 2.5% FCS, they grow at a much slower rate but are capable of being stimulated to grow more rapidly by a number of growth factors. Platelet-derived growth factor-BB, basic fibroblast growth factor and interleukin-1 alpha are all capable of increasing the growth rate of the SMCs above that of cells in the 2.5% FCS medium alone.

Foetal calf serum is an undefined medium and contains various bovine growth factors including PDGF, bFGF, and IGF-1. In the tissue culture medium with only 2.5% FCS, there are not enough of these growth factors present to allow significant proliferation of the vascular SMCs, but there are just enough to prevent programmed cell death and permit a slow turnover. The addition of exogenous PDGF, bFGF and IL-1α was able to stimulate proliferation of the SMCs by allowing synergy with the growth factors already present in the FCS. All 3 of these growth factors are produced by human saphenous vein endothelium. Basic FGF and IL-1α can also be produced by the SMCs, and HSVSMCs are capable of producing PDGF-AA, though probably not PDGF-BB. Thus any one of these growth factors could be present in the coculture experiment previously described (Allen *et al.* 1994). However, PDGF produced by far the greatest stimulatory effect on the cultured HSVSMCs. Interleukin-1α produced a relatively small increase in cell growth. Its action is largely due to induction of the endogenous production of bFGF (Sawada *et al.* 1990, Gay *et al.* 1991) and PDGF (Raines *et al.* 1989) by the SMCs. Basic FGF was a more potent stimulant of SMC proliferation in this experiment than IL-1α. However, bFGF lacks a signal sequence and is unlikely to be released by an intact vein in the coculture petri dish, unless there is cell damage or death. Whilst some cell death is

possible in culture, and bFGF may be released into the culture medium, the proliferative effects of bFGF on the cultured HSVSMCs were not as dramatic as those of PDGF. Therefore further study was directed towards the action of PDGF on HSVSMC proliferation in this organ culture model of intimal hyperplasia.

Platelet-derived growth factor is a well characterised mitogen for SMCs. Whilst the -A and -B chains are 60% homologous in their amino acid sequence (Westermark *et al.* 1989), they are encoded for by separate genes on different chromosomes (Ross 1989). The ability of the -B chain to stimulate both the A/B and the B receptors whilst the -A chain is only able to stimulate the A receptor does mean that there are some differences in the actions of the 2 growth factors. Koyama et al., working with cultured baboon aortic SMCs, found that stimulation of both PDGF receptors led to proliferation of SMCs but that migration of SMCs was only promoted by stimulation of the A/B receptor (which they referred to as the 'beta' receptor) (Koyama *et al.* 1994). Conversely, stimulation of the A receptor (which they referred to as the 'alpha' receptor) actually inhibited migration of the cultured SMCs. They also found PDGF-BB to have a greater proliferative effect on SMC growth than PDGF-AA. Hosang et al. also showed a lesser proliferative effect of PDGF-AA on human umbilical vein SMCs compared to PDGF-BB (Hosang *et al.* 1989a) whilst D'Amore et al. found a similar result with bovine aortic SMCs (D'Amore *et al.* 1993). Sachinidis et al. demonstrated a lower rate of thymidine incorporation with cells stimulated by PDGF-AA compared to PDGF-BB (Sachinidis *et al.* 1990). In addition, they were able to show that the 2 different receptors acted via different intracellular mechanisms. The B receptor caused an increase in inositol triphosphate and diacylglycerol production and a rise in intracellular calcium. The A/B receptor also generated diacylglycerol but with different kinetics and without the production of inositol triphosphate or a rise in intracellular calcium. Koyama et al. found that PDGF-AA inhibited SMC migration, an effect that was blocked by staurosporine, a protein kinase C inhibitor (Koyama *et al.* 1992). This suggests a role for protein kinase C in the action of the A receptor.

The response of cells to PDGF-AA depends upon the number of A receptors present in the cell type. Bovine aortic SMCs possess virtually no A receptors and consequently PDGF-AA has no mitogenic effect on these cells (Grotendorst *et al.* 1991). However, they can be

stimulated to produce more A receptors by bFGF (Schollmann *et al.* 1992). Under such circumstances, they will respond to PDGF-AA. Human saphenous vein SMCs may well possess more A receptors than bovine SMCs in culture as they do respond to PDGF-AA. Human aortic (Kanthou *et al.* 1992) and human saphenous vein SMCs (Winkles *et al.* 1991) can both be induced to increase production of PDGF-AA, which will lead to an autocrine proliferative effect as these cells possess A receptors. It would appear therefore that in humans both PDGF-AA and PDGF-BB are important in the proliferation of saphenous vein SMCs.

Chapter 6 - The role of Platelet-Derived Growth Factor in an organ culture model of human saphenous vein

6 a) THE EFFECT OF PDGF ON DE-ENDOTHELIALISED HUMAN SAPHENOUS VEIN IN AN ORGAN CULTURE

i) INTRODUCTION

ii) METHODS

iii) RESULTS

iv) CONCLUSIONS

6 b) THE EFFECT OF A NEUTRALISING ANTIBODY TO PDGF ON A COCULTURE MODEL OF DE-ENDOTHELIALISED AND INTACT HUMAN SAPHENOUS VEIN

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6 c) THE EFFECT OF CONDITIONED MEDIUM FROM HUMAN SAPHENOUS VEIN ORGAN CULTURE ON 3T3 FIBROBLAST CELLS

i) INTRODUCTION

ii) METHODS

iii) RESULTS

iv) CONCLUSIONS

6 a) THE EFFECT OF PDGF ON DE-ENDOTHELIALISED HUMAN SAPHENOUS VEIN IN AN ORGAN CULTURE

6 a.i) INTRODUCTION

The endothelium is no longer regarded as merely a passive diffusion barrier. It has a number of important physiological functions such as control of vascular tone and regulation of SMC proliferation. The endothelium is able to sense changes in shear stress and mechanical stretch (Davies 1991) and alters vascular tone accordingly (Rubanyi *et al.* 1990, Ando *et al.* 1993a). It produces a number of vasoactive substances. Endothelial-Derived Relaxing Factor, now known to be nitric oxide or a nitric oxide donor (Buga *et al.* 1991), and prostacyclin (Iba *et al.* 1991a) induce relaxation of the vasculature whilst endothelin is one of the most potent vasoconstrictors known to man (Yanagisawa *et al.* 1988). Overall vascular tone is the resultant of the effects of these opposing factors (Luscher *et al.* 1993).

The endothelium also shows a duality of function regarding SMC proliferation. As mentioned in Chapter 2, the endothelium is able to both inhibit and stimulate vascular SMC proliferation. An intact endothelial layer acts as a barrier to the adhesion of blood platelets and to the effects of serum mitogens. Under altered flow conditions, the endothelium itself will produce mitogens, notably PDGF (Hsieh *et al.* 1991) and endothelin (Kuchan *et al.* 1993, Wang *et al.* 1993) which are capable of stimulating SMC proliferation. In the vein culture model initially described by Soyombo et al. (Soyombo *et al.* 1990), the intimal proliferation of SMCs does not occur if the endothelium is denuded before the vein is placed in culture (Angelini *et al.* 1991). This implies a function for the endothelium in this model of intimal hyperplasia. The work by Allen et al. in our own department confirms this but also shows that coculturing a denuded segment of vein with an intact segment of vein will cause intimal proliferation to occur in the denuded segment of vein (Allen *et al.* 1994).

As this proliferation in the denuded segment of vein was caused by the intact vein, I decided to look at the effect of PDGF-BB, a mitogen produced by endothelial cells, on SMC proliferation in the denuded segment of vein. As shown in Chapter 5, PDGF-BB is a potent

mitogen for human saphenous vein SMCs. It is known to be produced by human vascular endothelium and may be liberated in the coculture model.

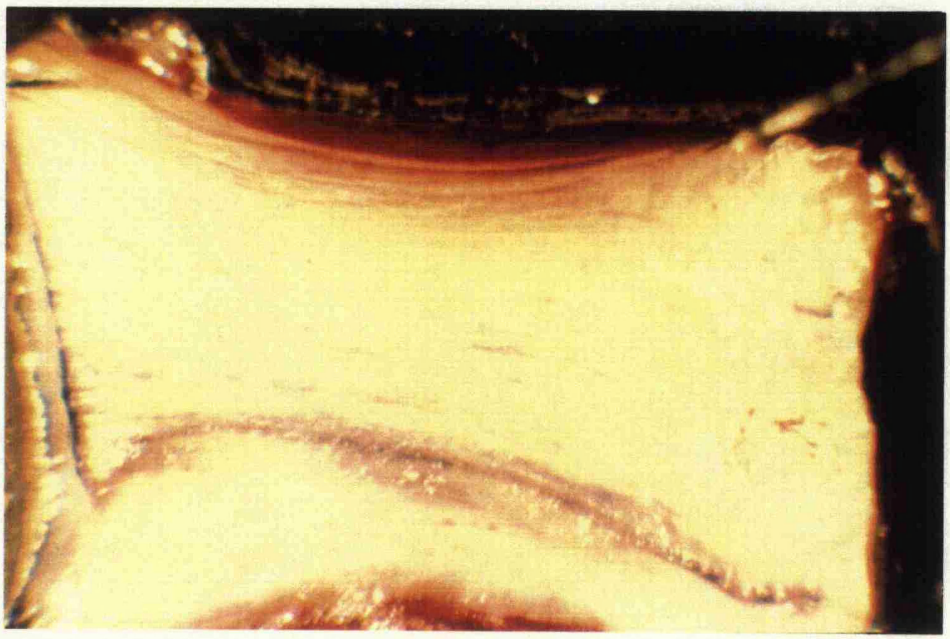
6 a.ii) METHODS

Local ethical committee approval was obtained to use redundant segments of human saphenous vein left over after aortocoronary or peripheral arterial bypass surgery. The segment of vein used for culturing was exposed using a no-touch technique and was dissected out with minimal handling. Tributaries were ligated with 3/0 silk. The length of vein available depended on the clinical requirements of the patient undergoing bypass surgery and was usually 2-3cm. The vein was not distended and was placed in sterile calcium-free Kreb's physiological saline solution immediately and transported back to the laboratory on ice. These precautions help to minimise trauma to the venous endothelium and reduce spasm of the vessel.

In the laboratory, the vein was placed in a sterile petri dish with the sterile calcium-free Kreb's solution in a laminar flow hood. Excess fat and adventitial tissue were removed using fine dissecting scissors and forceps. The vein was then opened longitudinally in the sterile medium. The immediate 2-3mm at each end of the vein were excised with a sharp size 23 scalpel blade and discarded as these pieces sustain some damage during harvesting and subsequent preparation. A 5mm length was then excised and tested for endothelial integrity. This was assessed using a trypan blue exclusion method. A solution of 0.2% trypan blue (Sigma Chemicals, Poole, Dorset) in 0.9% saline was pipetted over the luminal surface of the vein. This was left on for 1 minute and then washed off with physiological saline. The intact endothelium excludes the dye whilst damaged areas take it up and are stained blue. The percentage area of the vein which remained white (i.e. intact) was assessed by viewing the vein under an operating microscope. This has proven to be a quick and reliable method for assessing endothelial integrity and has been used extensively in our department (Allen *et al.*1994) as well as by other workers (Pederson *et al.*1985, Soyombo *et al.*1990). Figure 6a.1 demonstrates a segment of human saphenous vein with good endothelial integrity. In vein culture experiments requiring intact segments of vein, the vein was discarded if endothelial coverage was less than 50%. In practice, endothelial integrity varied from almost 0% to close

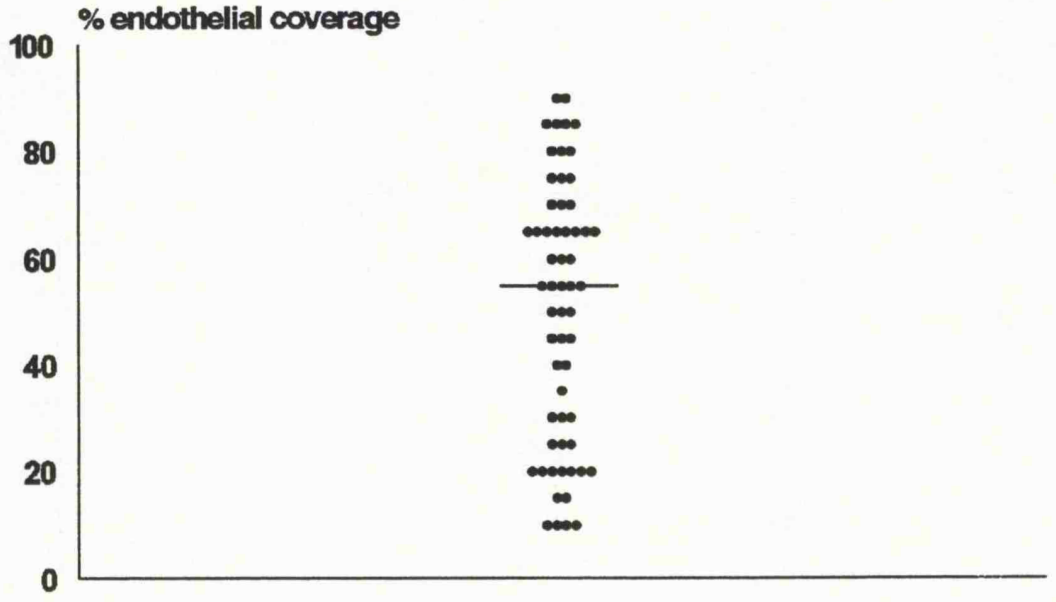
to 100%. In a consecutive series of 62 veins received from patients undergoing aortocoronary bypass surgery, the median endothelial coverage was 55% with a range of 10-90%. (Figure 6a.2)

Figure 6a.1 Segment of human saphenous vein stained with trypan blue. The intact endothelium excludes the dye which is taken up by damaged tissue.



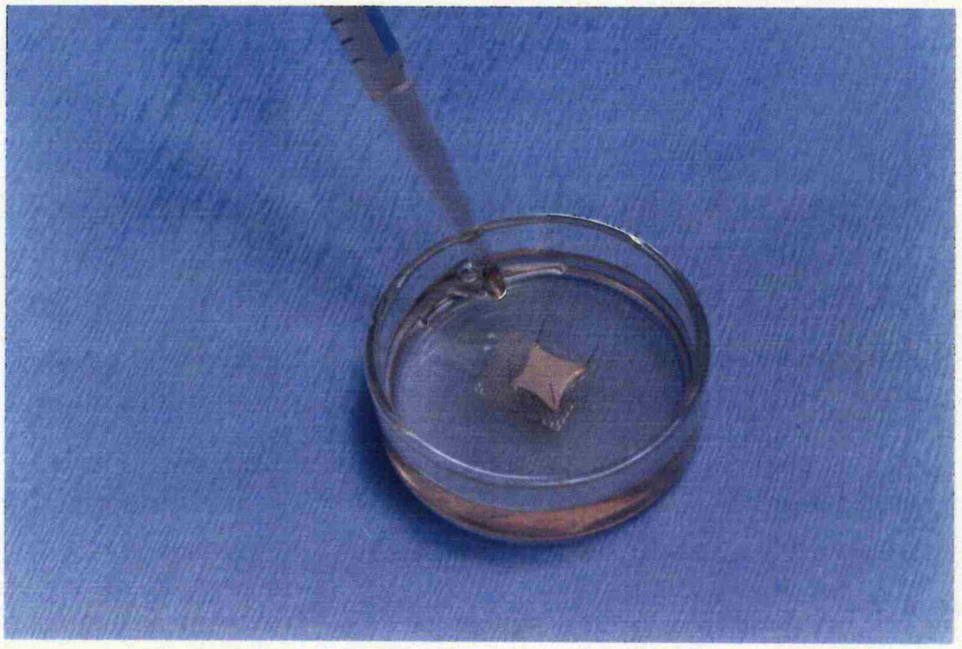
If the endothelial coverage was adequate (ie ≥50%), the remainder of the piece of vein was cut into appropriate-sized segments, approximately 5mm in length depending on the overall size of the vein. The veins were cultured in specially designed vein culture dishes. These consisted of 60 x 20mm pyrex dishes (Corning Ltd., U.K.) containing a layer of Sylgard 184 resin (Dow Corning, Seneffe, Belgium) approximately 5mm in depth. The vein was opened out with its luminal surface uppermost, placed on a coarse polyester mesh (500μm) and pinned into the resin with four A1 minuten pins (Watkins and Doncaster, Cranbrook,

Figure 6a.2 Scatter plot of endothelial coverage of 62 consecutive segments of vein obtained from patients undergoing aortocoronary or peripheral arterial bypass surgery.



Kent), one at each corner (Figure 6a.3). The vein culture medium was then added. A volume of 6mls was required to adequately cover the vein. The medium used was RPMI 1640 (Northumbria Biologicals, Cramlington, U.K.) containing 30% foetal calf serum (FCS) (Seralab, Crawley-Down, Sussex), L-glutamine at 2mmol/l, Penicillin 50U/ml and streptomycin 50μg/ml (all Northumbria Biologicals, Cramlington, U.K.). The vein cultures were kept in a humidified incubator (Queue Systems, West Virginia, U.S.A.) at 37°C, gassed with 5% CO_2 in air. The medium was replaced with 6mls of fresh medium every second day for a 14 day period. All segments of vein were placed in culture and in the incubator within 2 hours of harvesting from the patient.

Fig 6a.3 Vein segment in culture dish, pinned into Sylgard resin using A1 minuten pins.



At the end of the 14 day period, the culture medium was removed and the veins were fixed for staining, still pinned out in their dishes to prevent any shrinking. The fixative used was 10% formaldehyde in 0.9% saline ("Formal saline"). The veins were then dehydrated in stages using 70% through to 100% ethanol, cleared in chloroform and embedded in paraffin wax. Sections of 4μm thickness were taken from the paraffin blocks, mounted on silane coated glass slides and stained appropriately. The stains used routinely were Haemotoxylin and Eosin (H+E), a combined Miller's elastin and monoclonal smooth muscle actin stain, a monoclonal endothelial marker - CD 31 (Dako, High Wycombe, Bucks. U.K.) and a monoclonal antibody to stain the thymidine analogue 5-bromo-2-deoxyuridine (Brd-U). The H+E stain showed the general structure of the cultured vein. Collagen and elastin stained pink whilst cell nuclei stained blue. The combined Miller's elastin and monoclonal smooth muscle actin stain was developed in our histopathology department to best show the intimal hyperplasia in the cultured veins. The smooth muscle is stained light brown by the monoclonal smooth muscle antibody so that the neointima is stained a light brown colour. Elastin is stained black by the Miller's elastin stain and this provides a contrast to the neointima. The monoclonal antibodies

to CD31 (the endothelial marker) and to Brd-U respectively stain venous endothelial cells and proliferating cells a light brown colour.

The neointimal thickness for each vein was calculated by measuring the thickness of the neointima at a minimum of 30 points and taking the mean value. The measurements were made using a computerised image analysis system (Kontron Videoplan, Munich, Germany). The reproducibility of the technique was assessed in 10 veins using 2 separate observers (Mrs K.E. Porter and myself). In addition to measuring the neointimal thickness, a proliferation index was also assessed. This was calculated by counting all the neointimal cells which took up BrdU and all those cells which did not take up BrdU. The proliferation index was the number of cells stained by BrdU divided by the total number of neointimal cells. The cells were counted along the entire length of each vein (excluding the cut edges, where proliferation was generally increased) at x400 magnification under the microscope. This measurement was only useful if there was a significant neointima. If there were only a few cells present in the neointima, the proliferation index could be inaccurate as quite often those cells that were present did take up BrdU. Therefore measurements of the proliferation index were only really valid if the neointima was greater than 5μm thick.

To look at the effect of PDGF on a denuded segment of vein, paired segments of vein were used. Three pieces of vein were required for culture from each segment of vein obtained from a patient. Two pieces had the endothelium removed, one acting as a control and one added to the PDGF. The remaining piece of vein was left intact to use as a positive control. The endothelium was removed by pinning the vein in a culture dish and gently rubbing it with a sterile cotton wool bud. Previous work from our department has demonstrated that this action will remove the endothelium without causing significant damage to the underlying media. All 3 pieces of vein were cultured in vein culture medium supplemented with 30% FCS. One of the denuded segments of vein had PDGF-BB at 10ng/ml added to the culture medium. New PDGF was added at the same concentration at each change of vein medium. The veins were cultured for 14 days with a change of medium at every 2-3 days. At 72 hours prior to the end of the culture period, the labelling reagent Brd-U (Amersham, Bucks, U.K.) was added. This

thymidine analogue was taken up by proliferating cells and could be subsequently detected in the cultured vein.

At the end of the 14 day period, the veins were fixed and stained as described above. A total of 8 paired experiments were performed. The neointimal thickness and the percentage of proliferating cells stained with Brd-U were measured for each vein segment. The values were compared using a non-parametric test for paired samples - the Wilcoxon signed ranks test.

6 a.iii) RESULTS

There was variability between veins from different patients. Some representative examples of the histology of the veins after culture are shown in Figures 6a.4-6a.11. The ranges in neointimal thickness and proliferation index are shown in the scatter plots in Figures 6a.12 and 6a.13. The median [range] neointimal thickness for the intact segments of vein was 27.3μm [18.7-39.3μm], with a median [range] proliferation index of 30.2% [15.4-34.3%]. As might be expected, the denuded control vein had considerably less neointimal thickening. The median [range] thickness of the neointima was 1.1μm [0-3.5μm]. The denuded segment of vein with the PDGF had a median [range] neointimal thickness of 1.3μm [0-4μm]. As none of the denuded segments of vein developed a neointima greater than 5μm thick, their proliferation indices could not be measured accurately.

The differences in intimal thickness between the intact and the denuded segments of vein and between the intact and denuded segments of vein with PDGF were highly significant ($z = -2.5$, $p = 0.01$, Wilcoxon matched pairs signed-ranks test). The difference between the denuded segments and the denuded segments with PDGF was not significant ($z = -.17$, $p = 0.87$).

Figure 6a.4 Transverse section (x200) 'Intact' saphenous vein after 14 days of culture (H+E). There is an obvious neointima shown by the arrow.

Figure 6a.5 Transverse section (x200) 'Intact' vein after 14 days of culture (Smooth muscle and Miller's elastin). The neointima is clearly stained light brown (arrow).

Figure 6a.6 Transverse section (x200) 'Denuded' saphenous vein after 14 days of culture (H+E). There is virtually no neointima present.

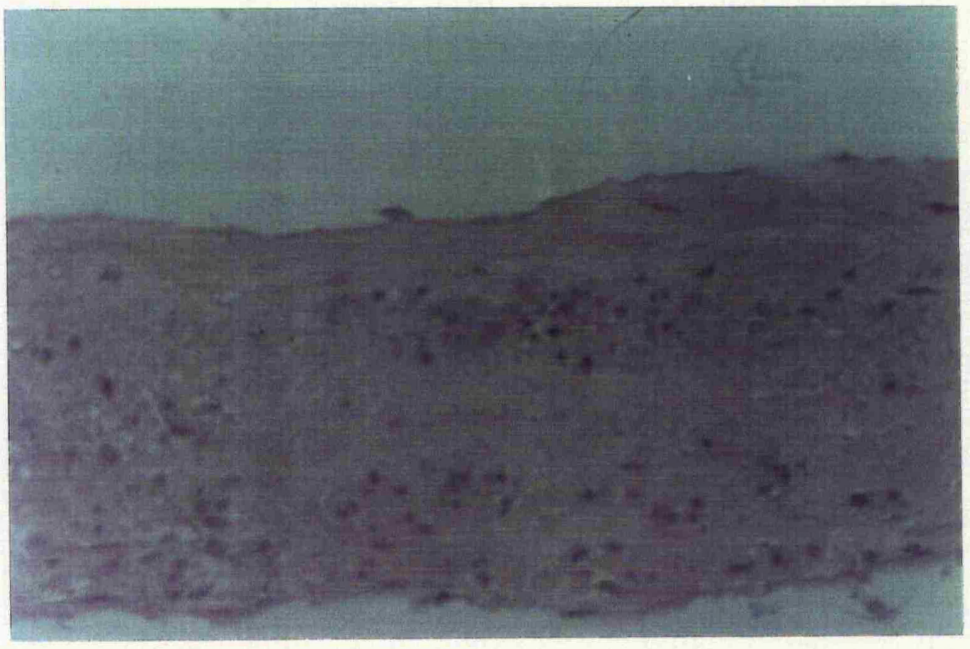


Figure 6a.7 Transverse section (x200) 'Denuded' saphenous vein after 14 days of culture (Smooth muscle actin and Miller's elastin). There is virtually no neointima present.

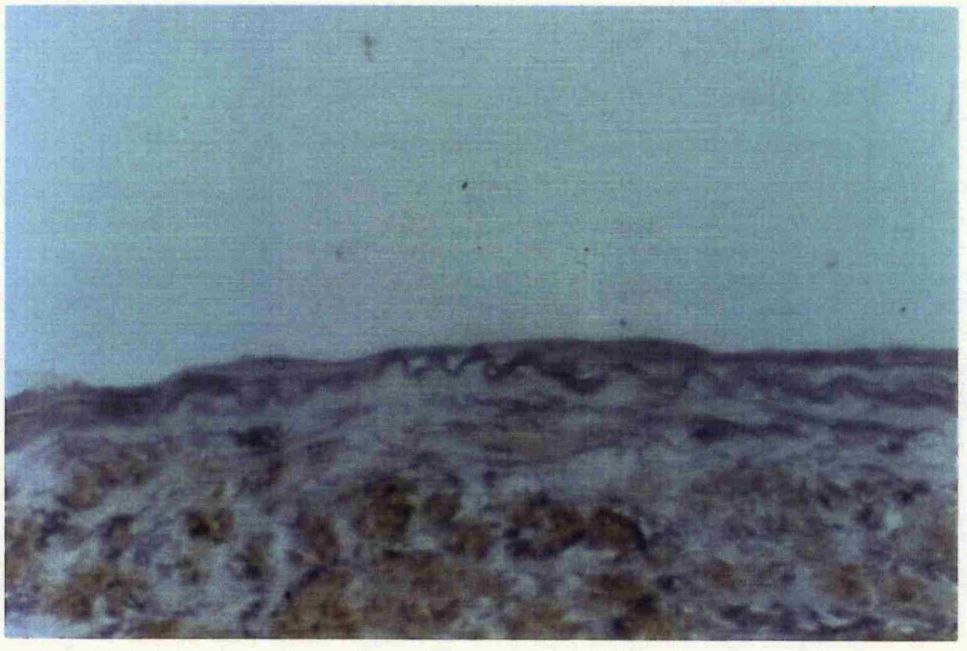


Figure 6a.8 Transverse section (x200) 'Denuded' saphenous vein with PDGF-BB after 14 days of culture (H+E). There is no real difference between this and Figure 6a.6, where there is no added PDGF.

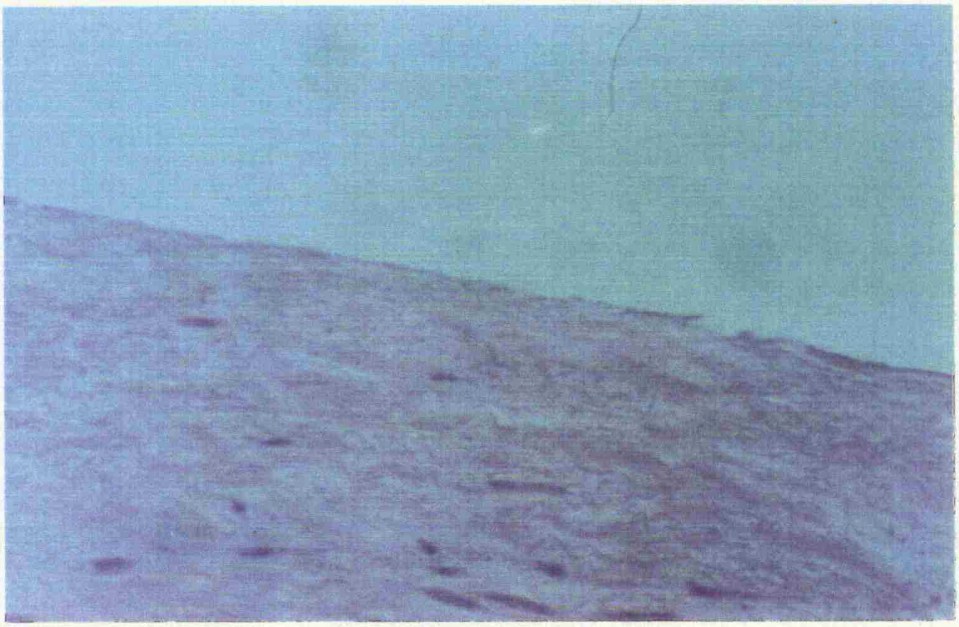


Figure 6a.9 Transverse section (x200) 'Denuded' saphenous vein with PDGF-BB after 14 days of culture (Smooth muscle and Miller's elastin)

Figure 6a.10 Transverse section (x400) 'Intact' saphenous vein after 14 days of culture (BrdU). The proliferating cells are stained brown.

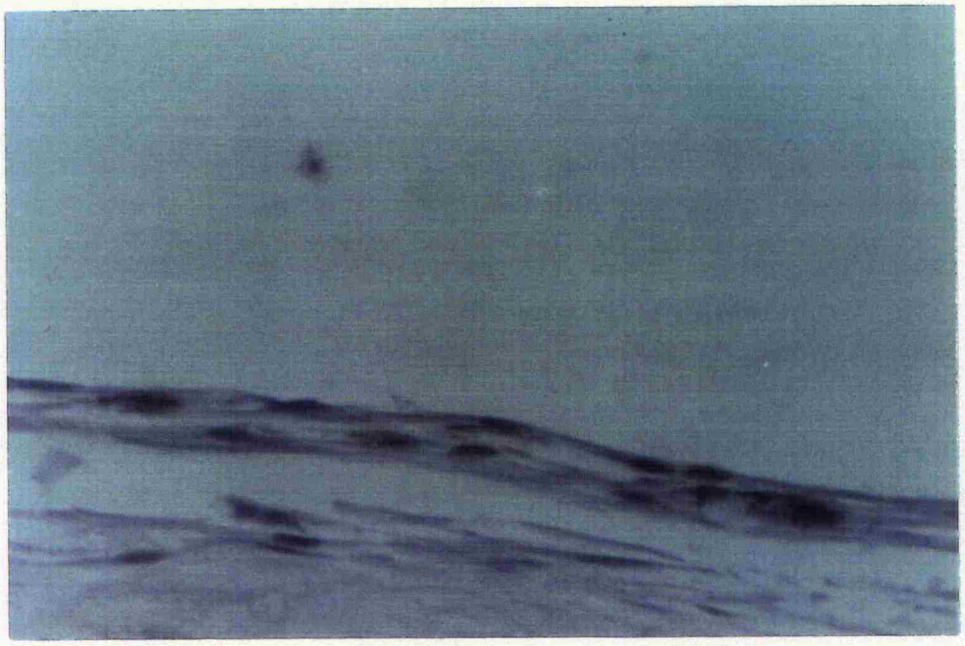


Figure 6a.11 Transverse section (x400) 'Denuded' saphenous vein with PDGF after 14 days of culture (BrdU). There are few stained cells in the intima.

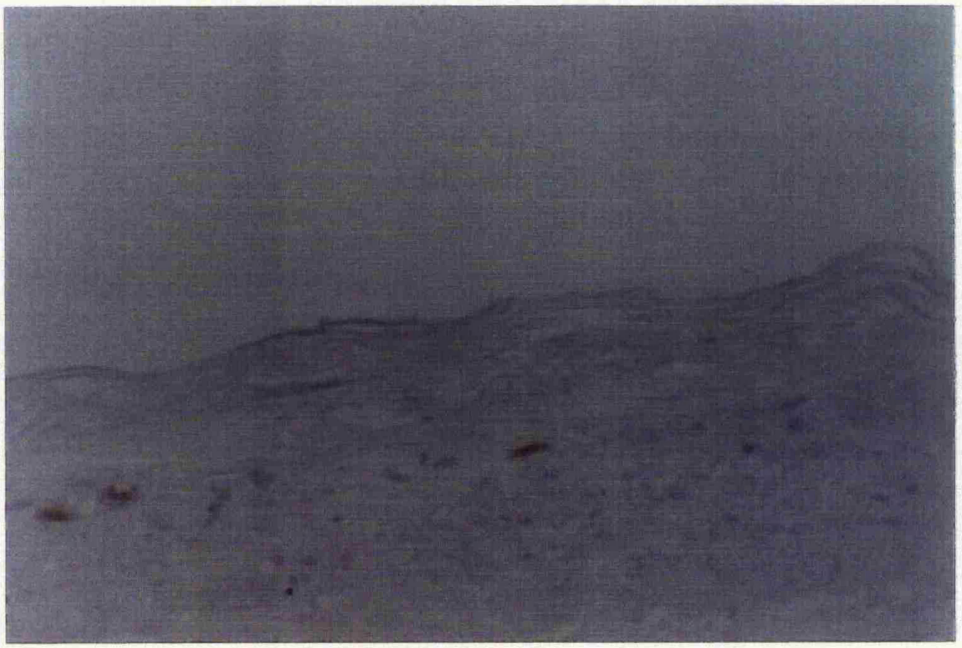


Figure 6a.12 Scatter plot of neointimal thickness of intact and denuded vein and denuded vein with PDGF. The median values are shown by a horizontal line.

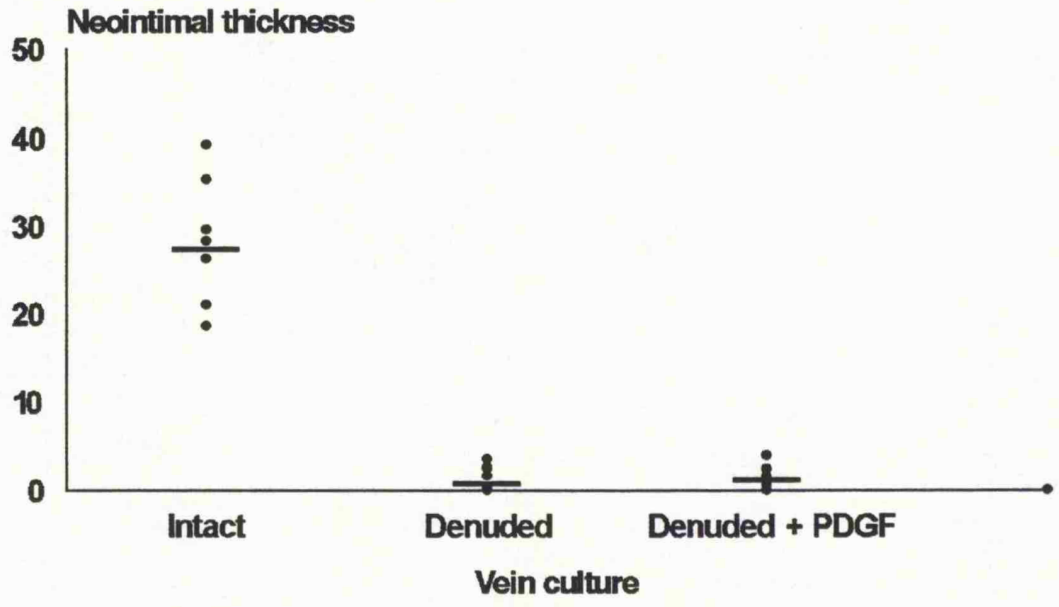
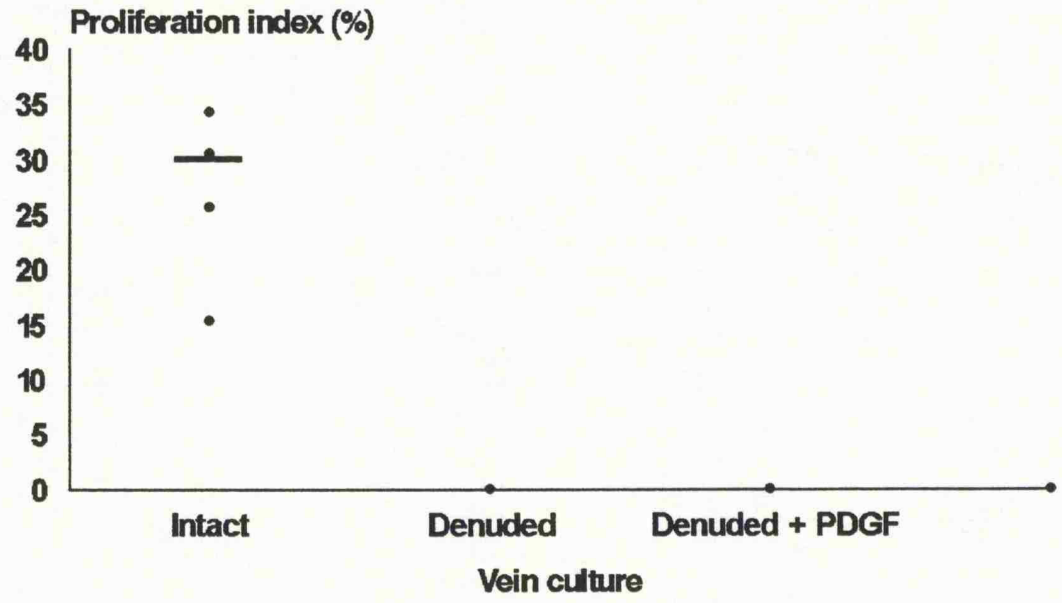


Figure 6a.13 Scatter plot of proliferation indices. The denuded segments of vein did not develop a significant neointima.



6 a.iv) CONCLUSIONS

Platelet-derived growth factor is a potent mitogen for human saphenous vein SMCs as demonstrated in Chapter 5. However, it is unable to stimulate the intimal proliferation seen in the neointimal cells of cultured human saphenous vein, when that vein is denuded of endothelium. There are several possible reasons for this. Whilst PDGF may be an important mitogen for SMCs, it is unlikely to be the sole stimulator of proliferation. The intimate contact of the endothelium with the SMCs in the intact vein allows paracrine mediation of this growth with very small quantities of growth factor. Direct cell to cell interaction may also help stimulate proliferation. However, the fact that proliferation will occur in a denuded segment of vein cocultured with an intact vein suggests that a paracrine mediator or mediators do exist which will stimulate SMC growth in the denuded vein. PDGF will not mimic this effect, so

there may well be factors released by the endothelium other than PDGF, which are not found in sufficient quantity in 30% FCS to stimulate proliferation. One likely candidate for this is endothelin. This was not tested in the initial SMC proliferation studies in Chapter 5, but is known to be mitogenic for vascular SMCs (Hirata *et al.* 1989), acts synergistically with PDGF (Weissberg *et al.* 1990) and has been shown by Masood et al (unpublished work) in our Department to be capable of stimulating proliferation of intimal SMCs in denuded segments of vein.

This study demonstrated that PDGF alone will not stimulate intimal proliferation in the denuded segments of vein, but it did not rule out the fact that PDGF may be important along with some other unidentified factor or factors.

6 b) THE EFFECT OF A NEUTRALISING ANTIBODY TO PDGF ON A COCULTURE MODEL OF DE-ENDOTHELIALISED AND INTACT HUMAN SAPHENOUS VEIN

6 b.i) INTRODUCTION

So far, I have shown that PDGF is a potent stimulator of human saphenous vein SMC proliferation in culture but that it is not able to stimulate proliferation of neointimal cells in a segment of cultured human saphenous vein which has had its endothelium removed. However, as already mentioned, previous work from our Department has shown that a denuded segment of vein cultured in the same petri dish as an intact segment of vein will develop intimal hyperplasia. In addition, conditioned medium from porcine aortic cultures is able to promote intimal hyperplasia in denuded segments of porcine aorta (Koo *et al.* 1989). Obviously, the intact segment of vein is not exerting its paracrine effect solely by the release of PDGF. The inability of PDGF to stimulate SMC proliferation in the denuded segment of vein does not completely exclude the possibility that PDGF may play some part in the process. It may act as a cofactor or a promoting agent. One way to see if PDGF does have a role is to use a blocking antibody to PDGF in the saphenous vein coculture to see if inhibition of PDGF alters the paracrine effect in this model.

6 b.ii) METHODS

In order to look at the effect of a blocking antibody to PDGF on the coculture model, a control coculture is required with no antibody and it was also decided to use a coculture with a non-specific antibody to rule out any non-specific effect on intimal hyperplasia by imunoglobulins. In addition to the coculture controls, a separate intact and a denuded control were also required so that a total of 8 segments of vein were required for each experiment. The high degree of variation between segments of vein from different individuals meant that all 8 segments of vein had to be obtained from the same patient. As each segment of vein for culture needs to be about 5mm in length, this meant a minimum of 4cm of vein was required (not including any discarded portions or portions required to assess endothelial integrity).

As described in 6 a), the lengths of vein were obtained from patients undergoing coronary or peripheral arterial bypass surgery. The lengths of vein were dissected out with a no-touch technique and tributaries were ligated with 3/0 silk. Once excised, the length of vein was immediately placed in a sterile universal tube containing calcium-free Kreb's physiological salt solution at 4°C and transported back to the laboratory on ice. The length of vein was dissected free of excess fat and adventitial tissue, the endothelial coverage was assessed and, if the vein was adequate, it was cut into 8 pieces of equal length using a sterile size 23 scalpel blade.

One segment was used as an intact control vein and one as a denuded control. The endothelium was denuded as described in 6 a) using a gentle rubbing action with a sterile cotton wool bud. The other 6 segments of vein were used in cocultures. Three coculture dishes were set up with one denuded and one intact segment of vein in each. All the veins were cultured for a 14 day period in standard vein culture medium containing RPMI 1640 with L-glutamine, penicillin and streptomycin supplemented with 30% FCS. In addition, one coculture dish contained a blocking antibody to PDGF whilst another contained a non-specific antibody.

The blocking antibody to PDGF was an anti-human PDGF neutralising antibody purchased commercially (R&D Systems Europe Ltd., Abingdon, Oxon., U.K.). The antibody is raised in goats against highly purified natural human PDGF and is able to neutralise human PDGF-AA, PDGF-AB and PDGF-BB. The product was supplied as a lyophilised powder which was reconstituted in sterile phosphate-buffered saline to provide a stock solution of 1mg/ml. Aliquots were frozen and stored at -20°C and used within 3 months. The antibody was used in culture at a final concentration of 10μg/ml, a concentration which was 2-3 times the ND_{50} for the antibody, according to neutralising assay data provided by the manufacturer.

The non-specific antibody was used at a similar working concentration of 10μg/ml. The antibody used was LDS-8, an antibody raised against human pancreatic exocrine tissue.

The vein culture medium was changed for all dishes on every 2nd day. In addition, fresh neutralising and non-specific antibodies were replaced at the same time. As in the experiment in 6 a), the veins were fixed at the end of the 14 day culture period with 10% Formal saline before being dehydrated in stages in alcohol, cleared in chloroform and embedded in paraffin wax. Sections were stained using the same stains as in 6 a).

The results were compared using the non-parametric Wilcoxon matched pairs signed ranks test. The statistical analyses were performed with the software SPSS for Windows (SPSS, Chertsey, Herts. U.K.) on an IBM-compatible personal computer.

6 b.iii) RESULTS

As in 6 a), the intact segments of vein developed a neointima after 14 days of culture whilst the denuded control vein did not. The coculture control veins developed some neointimal growth, but this was abolished by the neutralising antibody to PDGF. The non-specific antibody had no effect on the intimal hyperplasia. The intimal hyperplasia and the proliferation indices for the segments of vein are shown in Table 6b.1 below. There were a total of 8 experiments. The median values and the ranges are given in the tables. Scatter plots of the intimal hyperplasia and the proliferation indices are given in Figures 6b.1 and 6b.2.

The median intimal hyperplasia developed by the intact vein was 23.1μm. This did not differ significantly from that developed by the other intact veins in the coculture control (19.9μm, $z = -0.56$, $p = 0.58$), the coculture with the non-specific antibody (17.2μm, $z = 0$, $p=1.0$), or the coculture with the anti-PDGF (15.6μm, $z = -0.14$, $p = 0.89$).

Table 6b.1 Neointimal thicknesses and proliferation indices of cultured veins.

Vein	Intimal hyperplasia (μm) Median [Range]	Proliferation index (%) Median [Range]
Intact Control	23.1 [3.1-42.8]	28.7 [14.9-39.1]
Denuded Control	0 [0-21.5]	*-
Coculture Control		
Intact	19.9 [5.8-40.8]	27.2 [18.5-31.4]
Denuded	5.9 [0-13]	33.1 [19.3-42.9]
Coculture + non-specific antibody		
Intact	17.2 [7-26.1]	37.4 [22-46.7]
Denuded	5.8 [0-12.4]	28.4 [0-52.3]
Coculture + anti-PDGF		
Intact	15.6 [9.1-36.9]	29.6 [19.9-39.6]
Denuded	0 [0-5.9]	*-

*No proliferation index is given where the neointima is less than 5μm thick.

The intact vein did have significantly more intimal hyperplasia than the denuded vein ($z = -2.52$, $p = 0.012$), and the denuded veins in the coculture control ($z = -2.38$, $p = 0.017$), the coculture with non-specific antibody ($z = -2.37$, $p = 0.018$) and the coculture with anti-PDGF ($z = -2.52$, $p = 0.012$).

Whilst the coculture denuded vein did appear to develop more intimal hyperplasia than the denuded vein alone (5.9μm vs. 0μm), this did not reach statistical significance in this group of experiments ($z = -0.94$, $p = 0.35$). However, the coculture denuded vein did develop significantly more intimal hyperplasia than the denuded vein in the coculture with anti-PDGF ($z = -2.20$, $p = 0.028$). The denuded coculture vein with the non-specific antibody also developed significantly more intimal hyperplasia than the denuded coculture vein with the anti-PDGF ($z =$

-2.02, p= 0.043) but was not significantly different from the denuded coculture control (z = -0.10, p= 0.92).

Some representative examples of the histology of the cultured veins are shown in Figures 6b.3-6b.8.

Figure 6b.1 Scatter plot of intimal hyperplasia developed in the 8 segments of vein.

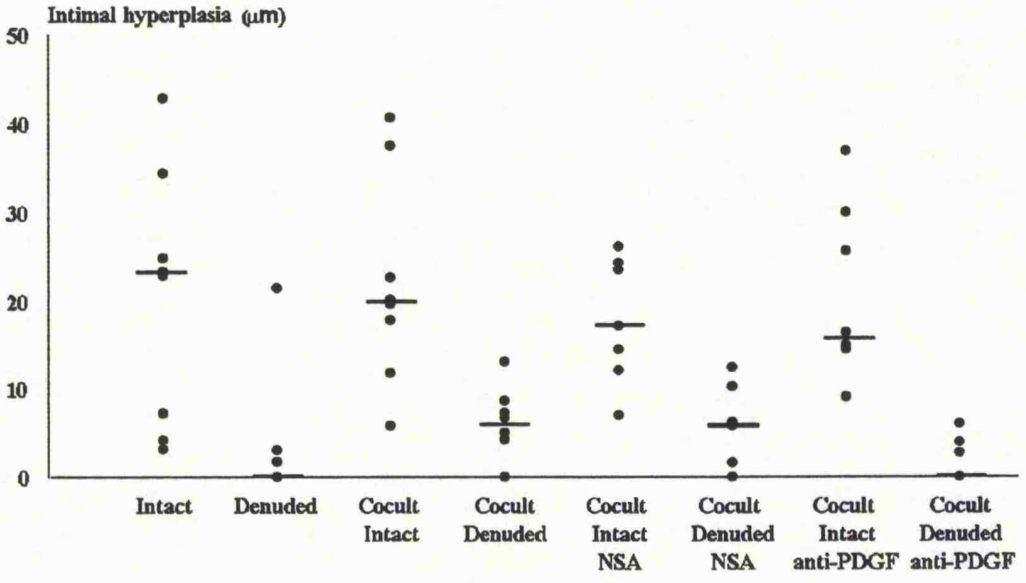


Figure 6b.2 Scatter plots of the proliferation indices of the 8 segments of vein. The neointimae in the denuded vein and the denuded vein in the coculture with anti-PDGF were less than 5μm thick.

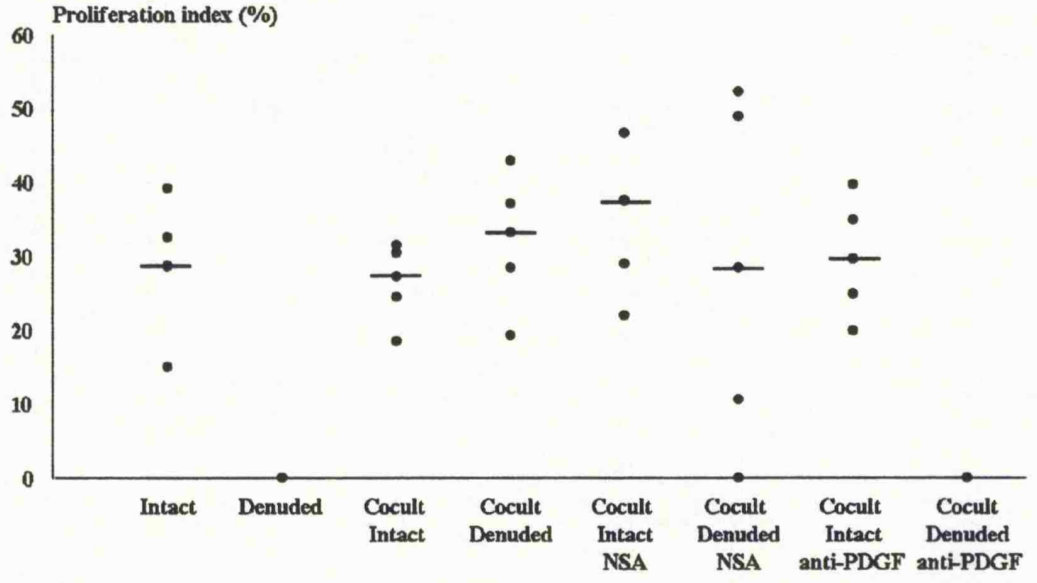


Figure 6b.3 Transverse section (x200) 'Intact' vein after 14 days of culture (Smooth muscle and Miller's elastin). The neointima is clearly stained light brown (arrow).

Figure 6b.4 Transverse section (x400) of intact control vein after 14 days of culture (Stained for BrdU). The proliferating cells are stained brown, as opposed to the blue nuclei of the non-proliferating cells.

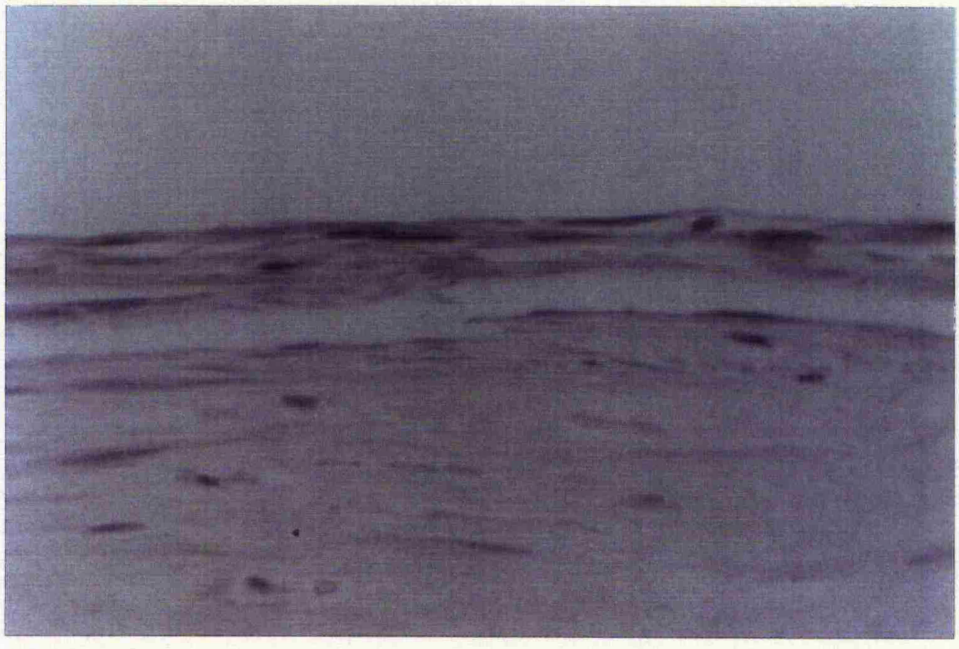


Figure 6b.5 Transverse section (x200) of denuded control vein after 14 days of culture (Smooth muscle and Miller's elastin). There is virtually no neointima present.

Figure 6b.6 Transverse section (x200) of denuded coculture control vein after 14 days of culture (Smooth muscle and Miller's elastin). The neointima is stained a light brown colour.

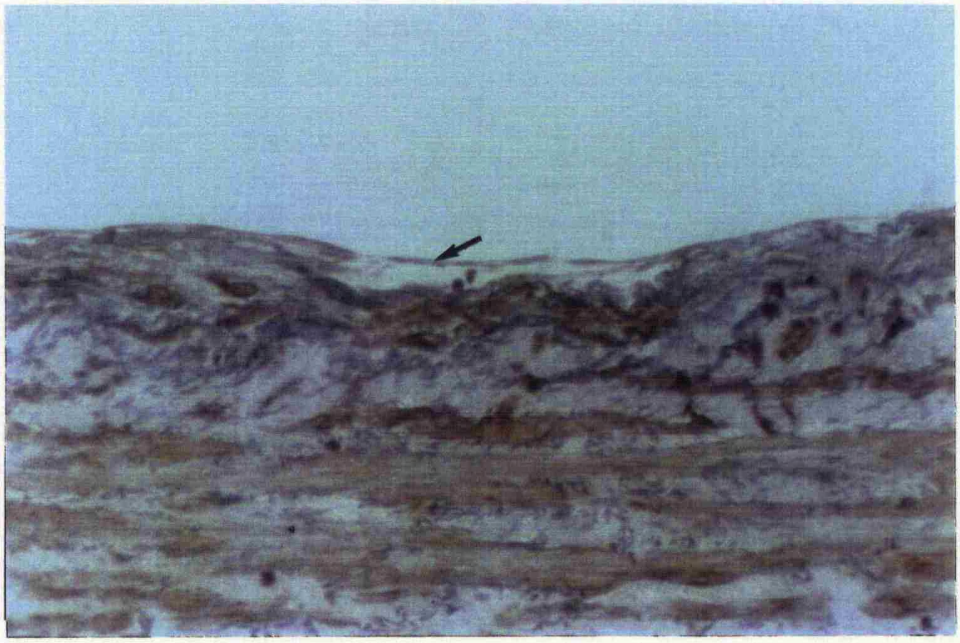


Figure 6b.7 Transverse section (x400) of denuded coculture control vein after 14 days of culture (Stained for BrdU). The proliferating cells in the neointima are stained a dark brown colour as opposed to the blue nuclei of the non-proliferating cells.

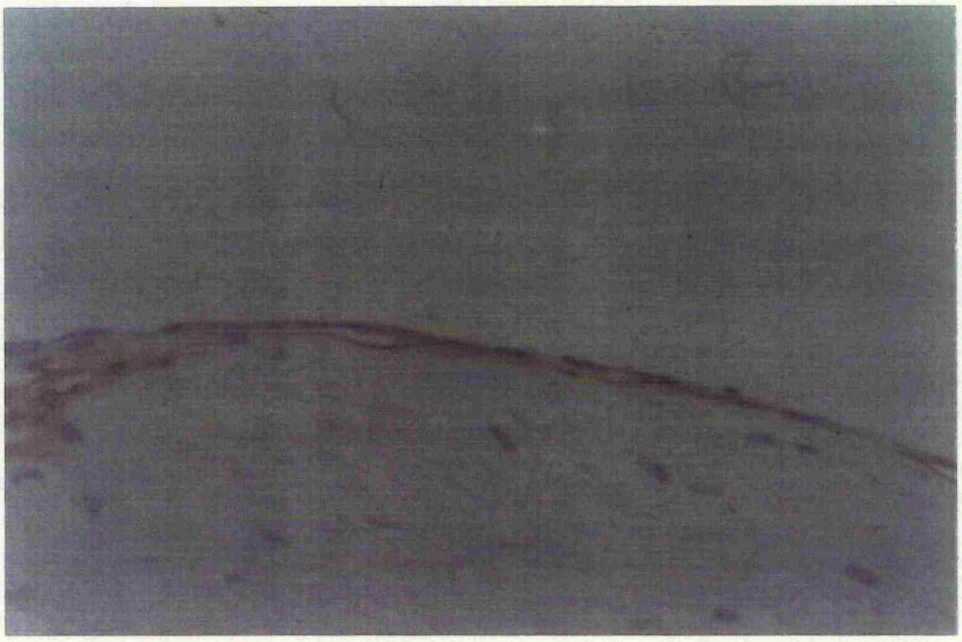
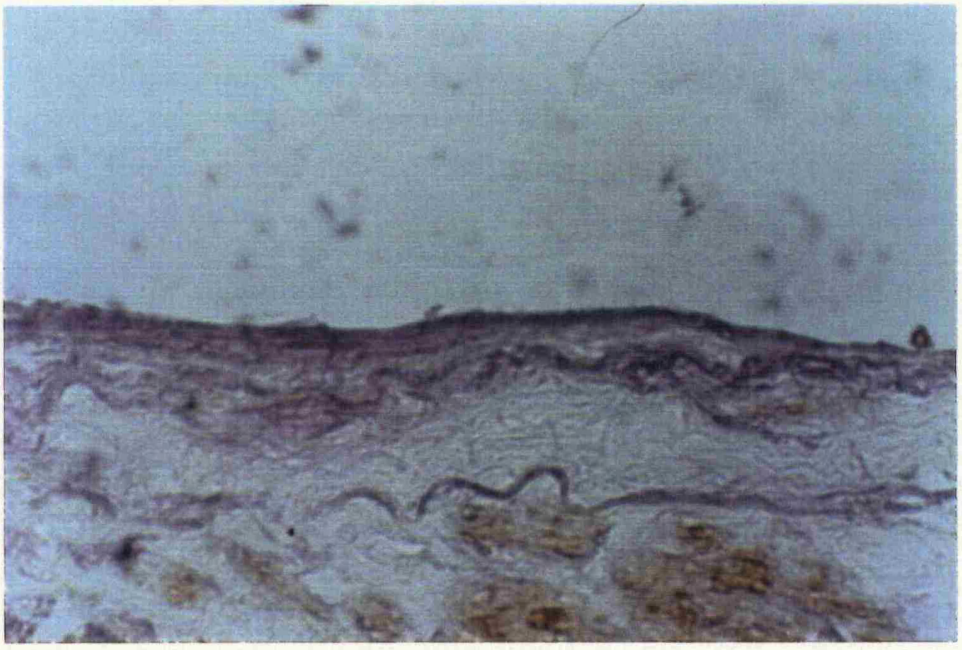


Figure 6b.8 Transverse section (x200) of denuded coculture vein with anti-PDGF antibody after 14 days of culture (Smooth muscle and Miller's elastin). There is no visible neointima.



6 b.iv) CONCLUSIONS

The ability of the antibody to PDGF to inhibit the intimal hyperplasia in the coculture model shows that PDGF has a role in the intimal proliferation in this model. The blocking antibody is either preventing bovine PDGF, present in the foetal calf serum, or PDGF released from the intact segment of vein from stimulating neointima development in the denuded segment of vein. The antibody has no effect on the intact segments of vein in the coculture, showing that the blocking of PDGF in the culture medium can not prevent the development of intimal hyperplasia in the intact vein. This may be because PDGF is released abluminally from the endothelium in the intact vein. It therefore does not come into contact with the anti-PDGF and is able to stimulate proliferation of the SMCs in the intima and media of the intact vein. The fact that PDGF, when added to the culture medium in 6 a), was unable to stimulate proliferation of the denuded segments of vein suggests that other factors must be present in the coculture which stimulate intimal SMC replication. It is likely that PDGF is required in this model along with another endothelial-derived factor, probably endothelin, to stimulate intimal proliferation in cultured human saphenous vein. Endothelin is known to require PDGF as a cofactor (Weissberg *et al.* 1990) and it may be that by blocking bovine PDGF in the medium or by blocking PDGF release into the medium from the intact vein, the proliferative effect of endothelin is being inhibited.

6 c) THE EFFECT OF CONDITIONED MEDIUM FROM HUMAN SAPHENOUS VEIN ORGAN CULTURE ON 3T3 FIBROBLAST CELLS

6 c.i) INTRODUCTION

The experiments in 6 a) and 6 b) show that intact saphenous vein is able to induce neointimal proliferation in a denuded segment of vein when cocultured in a medium containing 30% foetal calf serum, whilst the addition of PDGF to a denuded segment of vein has no effect. It would appear that the intact cultured vein is releasing a product or products which have a significant proliferative effect on human saphenous vein SMCs. This effect was blocked in the coculture model described in 6 b) by an antibody to PDGF. There were differences between the veins obtained from different individuals, both in their response to culture in the intact vein and in the ability of the denuded vein to proliferate. Other workers have commented on the differences between individual humans and the ability of their vascular cells to proliferate (Chan *et al.*1993). Human saphenous vein SMCs are therefore not an ideal cell type for studies which require reproducibility. One way to standardise the study of the proliferative effect of conditioned medium is to use a cell line that is reproducible. One such cell line is the 3T3 mouse fibroblast. These cells are responsive to PDGF but probably need a plasma factor, in addition to PDGF, to allow cell replication (Francis *et al.*1994). These cells have been used by several other workers as a biological assay of cell proliferation (Baird *et al.*1988, Limanni *et al.*1988, Hosang *et al.*1989a, Speir *et al.*1991, Sterpetti *et al.*1992b, Francis *et al.*1994). The use of blocking antibodies to inhibit proliferation in these cells is a useful technique to examine which components of a particular medium are responsible for proliferation.

6 c.ii) METHODS

A 3T3 mouse fibroblast line was cultured from a stock held in a liquid nitrogen bank in our Department. These cells were grown in culture medium consisting of RPMI 1640, supplemented with L-Glutamine 2mmol/l, penicillin 50U/ml and streptomycin 50μg/ml and 15% FCS (All Sigma Chemical Co. Ltd., Poole, Dorset). This is the standard medium used for culturing vascular SMCs and it proved very successful for culturing fibroblasts. The 3T3 cells

were subcultured as necessary. This was usually necessary once or twice per week, as the cells divided more rapidly than human saphenous vein SMCs.

'Conditioned' medium was obtained from culturing segments of human saphenous vein. Samples of vein were obtained as described in 6 a). These veins were dissected free of excess fat and adventitial tissue and, providing the endothelial coverage exceeded 50%, they were cultured for a period of 10 days. At the end of the 10 day period, the medium was removed, the veins were gently washed in situ with RPMI medium and then the veins were placed back in the incubator with 6mls plain RPMI. After 24 hours the RPMI was removed. This was put aside and used as the 'conditioned medium'. This medium was pooled then frozen and stored at -20°C for a maximum of 3 months. The decision to use conditioned medium after 10 days of culture was taken as previous work from our Department has shown maximum proliferation in the intimal SMCs is achieved at about 10 days. The production of any growth factors would therefore be expected to be high at this time.

When enough medium was available for an experiment, the 3T3 cells were plated out in 96 well plates in a serum-free medium, containing RPMI 1640 supplemented with L-Glutamine 2mmol/l, penicillin 50U/ml and streptomycin 50μg/ml but no foetal calf serum. The cells were plated out in 200μl aliquots at a concentration of 5×10^4 cells/ml, so that each 200μl aliquot contained 10^4 cells. The cells were kept in the serum-free medium for 48 hours to ensure that they remained relatively quiescent. After this time, the medium was replaced with either conditioned medium, conditioned medium with anti-PDGF at a final concentration of 10μg/ml (as used in 6 b)), standard culture medium with 15% FCS or plain RPMI.

Each well also had 1μCi of tritiated thymidine (methyl-^{3}H thymidine, Amersham Int PLC, Bucks, U.K.) added. This was supplied with a radioactivity of 1.0mCi/ml (37 MBq/ml) and was diluted in RPMI 1640 so that 10μl aliquots could be accurately added to each well using a precision syringe (Hamilton Bonaduz AG, Bonaduz, Switzerland) designated for use with radioactive materials. The wells were then left for 18 hours to allow the 3T3 cells to incorporate the thymidine. Other workers have used the same amount of tritiated thymidine

and pulsed the 3T3 cells for 18-20 hours (Sterpetti *et al.* 1992b, Francis *et al.* 1994) to study thymidine incorporation.

At the end of this period, the medium was gently aspirated and the cells were harvested. This was not always easy as the cells adhered to the bottom of the 96 well plates. Optimal harvesting was obtained by adding 200µl of Trypsin and EDTA, incubating for 10 minutes at 37°C, and then using a cell harvester. The cells were adsorbed onto printed glass fibre filter paper ('Printed Filtermat A', Wallac Oy, Turku, Finland) using a mach III harvester 96 (Tomtec, Orange, Conn, U.S.A.). The filter paper with the adsorbed cellular material was then placed in a 90cm x 120cm sample bag (Wallac Oy, Turku, Finland) and 3.5mls of liquid scintillant cocktail ('Betaplate Scint', Wallac Oy, Turku, Finland) was added and evenly distributed over the filter paper using a roller. The sample bag was then sealed and placed in a 96 well cassette so that the beta activity could be measured in a Wallac Microbeta Plus liquid scintillation counter (Wallac Oy, Turku, Finland). This machine has several pre-programmed settings including one for calculating beta emissions from tritiated thymidine. Activity was measured in counts per minute (cpm). Four wells were counted for each medium in each experiment and a mean value was taken. A total of 8 experiments were performed. The counts were compared using a Wilcoxon matched pairs signed ranks test, calculated using the statistical package SPSS for Windows (SPSS, Chertsey, Herts. U.K.) on an IBM-compatible personal computer.

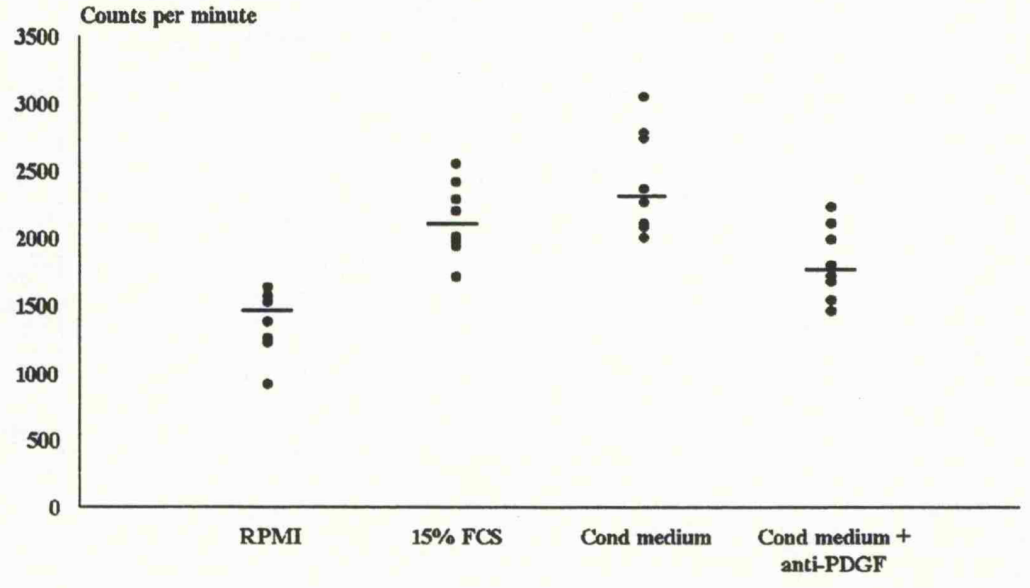
All radioactive materials, medium and equipment used were disposed of according to the University's guidelines on the handling of radioactive substances.

6 c.iii) RESULTS

The 3T3 cells in the plain RPMI produced a median [range] count of 1449 [920-1629]. This compares with a median [range] count of 2106 [1703-2549] for those cells in 15% FCS medium. The cells in the conditioned RPMI medium produced a median [range] count of 2316 [2003-3052], whilst those cells in the conditioned medium with the anti-PDGF antibody produced a median [range] count of 1755 [1457-2233]. The counts are represented in the scatter plot in Figure 6c.1.

The difference in proliferation between the 15% FCS medium and the plain RPMI was significantly different (z = -2.52, p= 0.012, Wilcoxon matched pairs signed ranks test) as might be expected. After all, the 3T3 cells had grown rapidly in this medium when they were being cultured, but had remained relatively quiescent in the serum-free medium. The conditioned medium produced a significant effect above that produced by the RPMI alone (z = -2.52, p= 0.012, Wilcoxon matched pairs signed ranks test). The conditioned medium also appeared to produce a greater effect than the 15% FCS medium, but this was not statistically significant (z = -1.54, p=0.12, Wilcoxon matched pairs signed ranks test). The addition of the anti-PDGF antibody to the conditioned medium significantly decreased the proliferative effect of the medium (z = -2.52, p= 0.012, Wilcoxon matched pairs signed ranks test) but, interestingly enough, the conditioned medium with the anti-PDGF still stimulated the 3T3 cells more than the plain RPMI alone (z = -2.1, p= 0.036, Wilcoxon matched pairs signed ranks test).

Figure 6c.1 Scatter plot of the counts produced by the 3T3 cells in the different media.



6 c.iv) CONCLUSIONS

The above results show that the cultured vein is able to release factors into solution which will cause 3T3 cells to incorporate tritiated thymidine. It is likely that the growth factors released are the same ones that cause the denuded segments of vein in the coculture model to proliferate. However, in this series of experiments, the addition of a blocking antibody to PDGF caused only a partial reduction in thymidine incorporation within the fibroblasts. The 3T3 cells in the conditioned medium with the anti-PDGF remained stimulated to a greater extent than the cells in the plain RPMI. The partial reduction after addition of the anti-PDGF suggests that there is PDGF in the medium but that other factors are also present which are capable of stimulating thymidine incorporation. This accords with the results in 6 a), where it was shown that PDGF alone was unable to stimulate the proliferation of neointimal cells in the denuded segment of vein whilst factors released from the intact segment of vein were able to stimulate SMC proliferation in the coculture model.

There is a difference between mere thymidine incorporation and actual cellular proliferation. The 3T3 cells need a whole host of factors to allow cell division and replication, but thymidine incorporation can take place with a bare minimum of nutrients. Thus the conditioned RPMI medium is adequate for thymidine incorporation but is unlikely to be sufficient for significant cell replication. Even after addition of the antibody to PDGF to the conditioned medium, there was still thymidine incorporation in the 3T3 cells. This contrasts with the coculture experiments in 6 b), where the addition of antibody to PDGF completely abolished the proliferation of vascular SMC in the denuded vein in the coculture. There are 2 explanations for this. Firstly, human saphenous vein SMCs may not respond to mitogens in an identical manner to 3T3 fibroblasts. Secondly, I have not shown that conditioned medium containing antibody to PDGF will cause proliferation of 3T3 fibroblasts, only that it will cause incorporation of tritiated thymidine. The growth factor or factors responsible may not be able to cause cell proliferation without PDGF or serum mitogens.

Chapter 7 - The development of an *in vitro* model of intimal hyperplasia incorporating arterial flow conditions

7 a) INTRODUCTION

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7 a) INTRODUCTION

The organ culture model of vascular tissue is a useful *in vitro* model of intimal SMC proliferation in both arteries (Fingerle *et al.* 1987, Koo *et al.* 1989) and veins (Soyombo *et al.* 1990). It is particularly useful as it allows a study of human tissue in a controlled, reproducible environment. The cellular processes involved in intimal hyperplasia in human vessels can therefore be studied, rather than those in animal tissue. This has several advantages, apart from the fact that it spares the lives of some laboratory animals. Organ culture has the advantage over cell culture that the cells are maintaining their normal intercellular relationships. It is therefore one step closer to the *in vivo* situation than isolated cell culture.

As mentioned earlier, the response of human vascular tissue to various stimuli differs markedly between individuals. Thus it is difficult to interpret the responses to different stimuli in different individuals. However, if several segments of tissue are obtained from the same individual, it is possible to expose each of them to different stimuli and to compare the effects of the different stimuli for that individual. In addition, organ culture allows the study of living human tissue, rather than animal tissue. Thus the cellular processes of intimal hyperplasia in human saphenous vein can be studied rather than an animal model such as the rat carotid artery. There are considerable interspecies differences in the response to various mitogens and growth inhibitors (Ferrell *et al.* 1992). As human saphenous vein is the conduit used in arterial bypass surgery, it makes sense to study this tissue rather than animal arterial tissue.

Whilst the *in vitro* study of cultured blood vessels does allow analysis of the processes involved in proliferation of human tissue, it remains artificial. Instead of having pulsatile oxygenated blood passing through the vessel lumen, the vessel is opened up and pinned out with its luminal surface uppermost in culture medium supplemented with 30% foetal calf serum. The static culture conditions may influence the intimal proliferation seen. In the human saphenous vein model, there is significant intimal hyperplasia after 14 days of culture (Soyombo *et al.* 1990), whilst the development of vein graft stenoses is not seen in the first month after surgery (Varty *et al.* 1993a). The absence of flow in the vein culture model may be partly responsible for the accelerated intimal hyperplasia. In animal models, an increase in shear

stress brought about by increasing flow leads to a reduction in intimal hyperplasia whilst a fall in shear stress produces an increase in intimal hyperplasia (Faulkner *et al.* 1975, Rittgers *et al.* 1978, Morinaga *et al.* 1985, Dobrin *et al.* 1989, Kraiss *et al.* 1991). Conversely, an increase in cyclic stretch produced by pulsatile blood within the vessel lumen stimulates SMC proliferation (Predel *et al.* 1992) and production of PDGF (Wilson *et al.* 1993) and leads to an increase in medial thickness *in vivo* (Dobrin *et al.* 1989). The effects of shear stress and cyclic stretch are obviously lost in the static vein culture model.

7 b) SETTING UP A FLOW MODEL.

7 b.i) METHODS

In order to simulate the effects of pulsatile flow in a blood vessel, it is necessary to reproduce both the flow and pressure effects. An approximation of arterial pressure is required, along with a flow equivalent to that in a femoropopliteal bypass graft. An appropriate medium is required to maintain the viability of the vein in culture. This medium needs to be maintained at constant temperature and pH, much as the medium in the static vein culture is maintained at a steady temperature, pH and O_2 saturation. Any tissue culture model also needs to remain sterile, as the growth of micro-organisms within the culture medium is likely to adversely affect the cells or tissue being studied.

A semi-closed system was employed as illustrated in the line diagram in Figure 7b.1. A modified conical flask was used as a reservoir for the culture medium (Figures 7b.2 and 7b.3). The flask was a standard pyrex flask which was adapted by the glass-blower in the Department of Chemistry at Leicester University. The flask had a fluid inlet and outlet incorporated into it. The fluid could be gassed by an air and CO_2 mixture via a gas inlet pipe in the stopper. A gas outlet pipe allowed excess gas to pass out. The presence of a gas outlet also allowed for fluctuations in the volume of fluid within the reservoir without excessive changes in pressure within the flask which would occur if the system was entirely 'closed'. Sterility was maintained by ensuring that the gas outlet pipe was protected by a 0.2μm bacterial filter ('Sterile Acrodisk', Gelman Sciences, Ann Arbor, Michigan, U.S.A.). The gas mixture was passed at a

rate that allowed a steady stream of bubbles to pass through the medium without disturbing it enough to cause excessive 'frothing' of the medium. Analysis of the medium using a blood gas analyser on the intensive care unit at the Leicester Royal Infirmary confirmed that the pH, PO_2 and PCO_2 were being maintained at adequate levels.

The medium used was standard vein culture medium consisting of RPMI 1640 (Northumbria Biologicals, Cramlington, U.K.) containing 30% foetal calf serum (FCS) (Seralab, Crawley-Down, Sussex), L-glutamine at 2mmol/l, Penicillin 50U/ml and streptomycin 50μg/ml (all Northumbria Biologicals, Cramlington, U.K.). In the static culture model, this medium was replaced every 2 or 3 days. This was not really practical from the flow model's point of view as it greatly increased the risk of infection. In addition, the volume in the static vein culture dish was only 6mls. Assuming this was changed every second day, this would mean a total volume of 7 x 6 = 42mls used for each vein in a static experiment. The volume of medium in the flow model was 500mls. In view of this, the vein is unlikely to run out of nutrients during the 14 days in culture and any toxic metabolites would be considerably diluted. Bromodeoxyuridine (Brd-U, Amersham, Bucks, U.K.) was added to the medium for the final 72 hours of culture to identify proliferating cells.

Figure 7b.1 Line diagram showing flow circuit. The direction of flow is indicated by the arrows. The medium is pumped from the reservoir into the vein chamber and back to the reservoir. The vein chamber is kept in an incubator (Gallenkamp, U.K.), and the silicone tubing and the reservoir are lagged with polystyrene to insulate them.

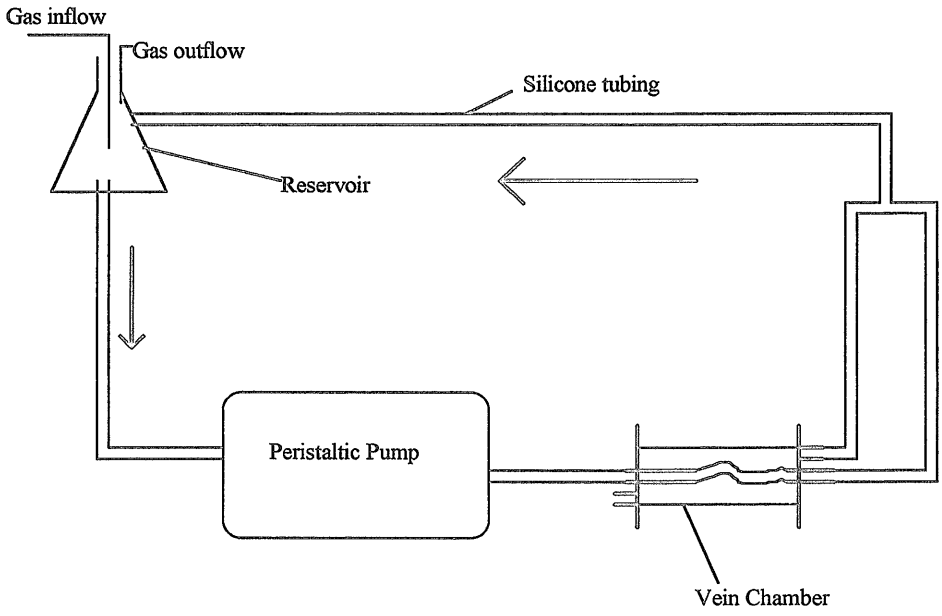


Figure 7b.2 Photograph of glass reservoir.

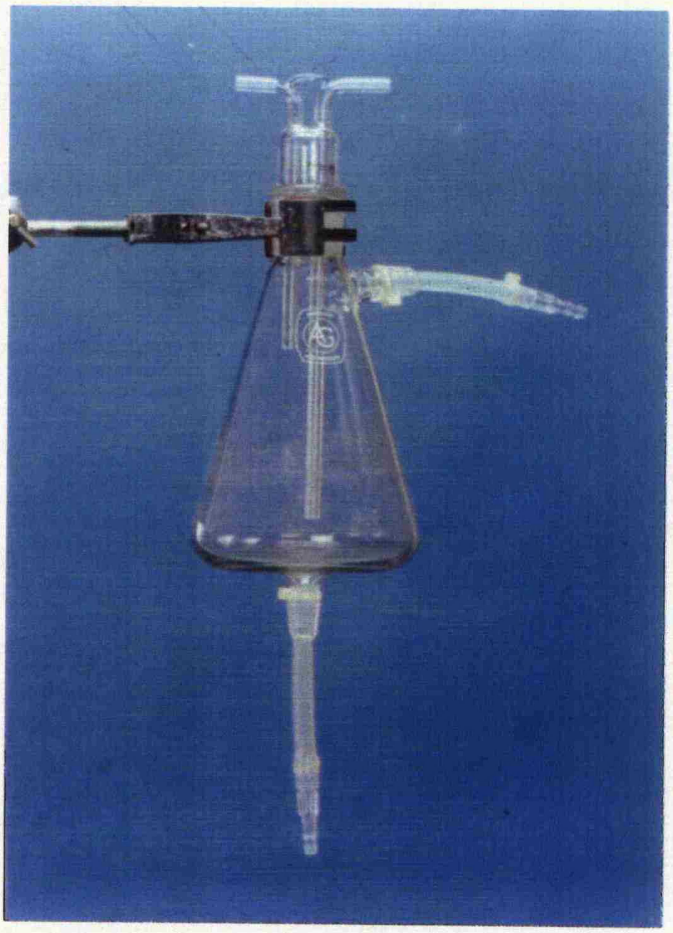
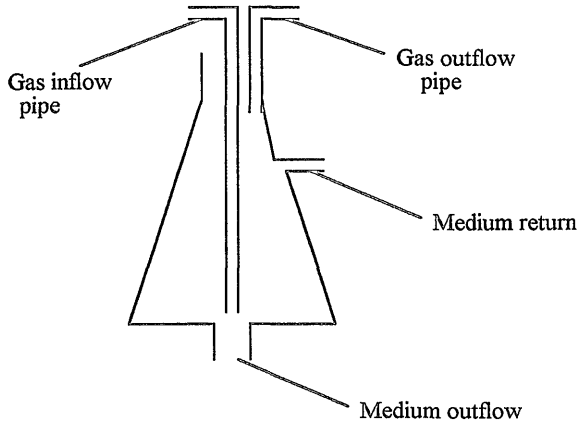


Figure 7b.3 Line diagram of reservoir showing medium outflow and return as well as gas inflow and outflow.



The vein was housed in a special vein chamber (Figures 7b.4 and 7b.5). This was an adapted endothelial cell seeding chamber previously used for the seeding of small diameter PTFE grafts with endothelial cells. The chamber was shortened for use with segments of saphenous vein. The chamber was composed of a threaded polymethylpentene tube (Just Plastics Ltd., London, U.K.) with a screw-on stainless steel cap at either end. The join between the cap and thread was sealed with a silicone rubber O-ring. Each cap had 2 portholes in it, one placed centrally and one off-centre. Through the central porthole it was possible to pass a stainless steel cannula via a watertight seal. The position of this cannula within the vein chamber could be adjusted whilst retaining a watertight seal. The off-centre porthole allowed access to the outer part of the vein chamber via a Luer lock connection.

Figure 7b.4 Vein chamber. This photograph shows a small diameter PTFE graft rather than a saphenous vein attached to the cannulae.

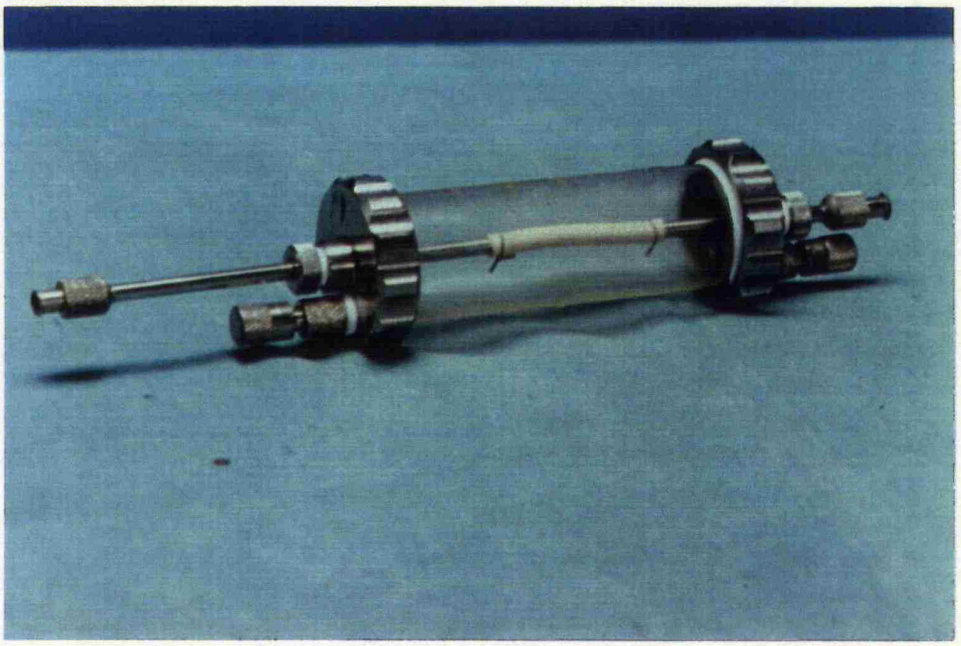
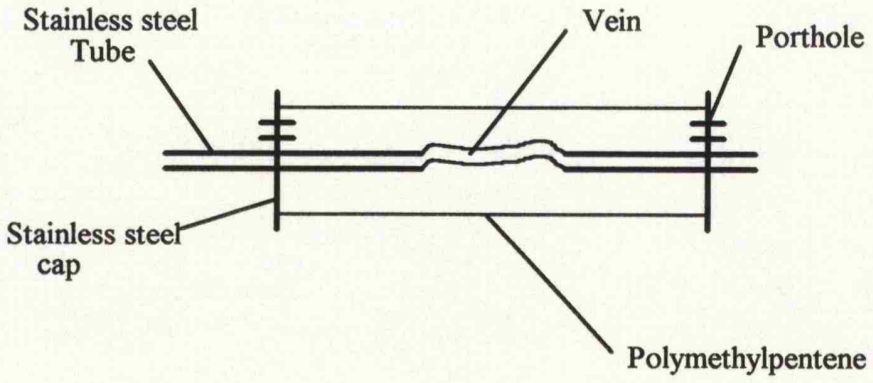


Figure 7b.5 Line diagram of the vein chamber demonstrating the stainless steel cannulae and the portholes.



Once the vein was sealed inside the vein chamber, and perfused with medium, it was still surrounded by air. The off set portholes could be used to top up the vein chamber with culture medium so that in addition to being perfused with culture medium, the vein segment

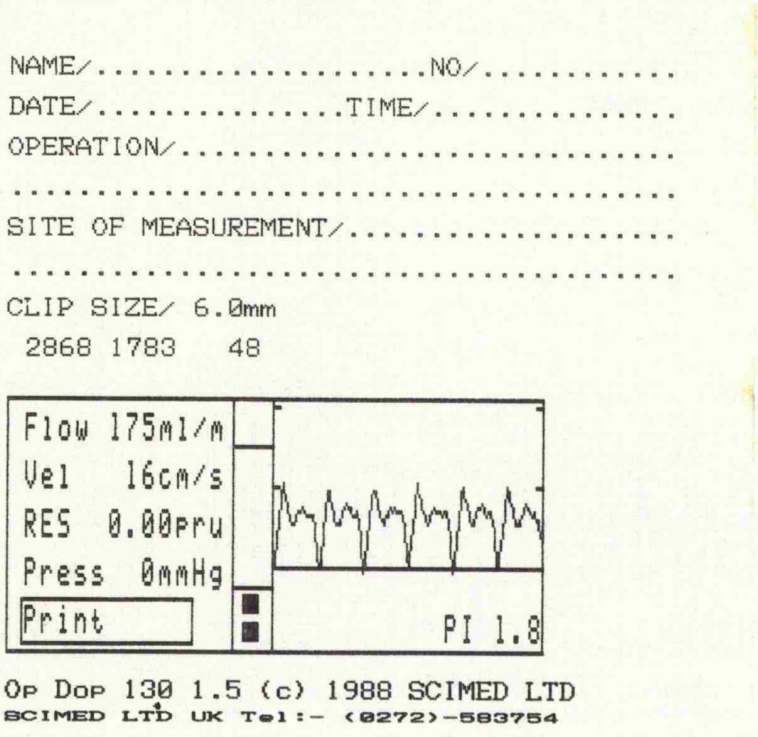
was also completely bathed in the medium. It was initially intended not to circulate any fluid around the outside of the vein, in order to keep the circuit as simple as possible. However, it became apparent that several of the leaks produced by this system were due to a significant rise in pressure within the vein chamber. Indeed, on occasions, the vein could be seen to kink and flow would decrease because of a rise in pressure within the chamber, presumably due to a leak of fluid from a small untied venous tributary into the vein chamber. This rise in pressure was avoided by connecting a separate piece of silicone rubber tubing to one of the off set portholes and attaching the other end to the fluid return tubing via a 'Y' connector. Whilst this did mean another two connections with a subsequent higher risk of infection, it did allow the flow rig to work more smoothly.

7 b.ii) MEASUREMENT OF FLOW AND PRESSURE PARAMETERS.

Pulsatile flow was provided using a Watson-Marlow 503U peristaltic pump (Watson-Marlow Ltd., Cornwall, U.K.) (Figure 7b.6). This pump can rotate at up to 50 revolutions/minute (RPM), producing 2 'pulses' for each rotation. Thus at 30 RPM, it produces 60 pulses per minute, akin to a heart rate of 60 beats per minute. The flow rate produced by the pump could be increased by using a faster rate or by using pump tubing with a wider diameter. Flow rate was measured in 2 ways. Firstly, an 'Op Dop' Doppler flowmeter (Scimed Ltd., U.K.) could be used to measure flow velocity and calculate flow rate providing that the fluid contained particulate material that would reflect the sound waves from the 'Op Dop'. Vein culture medium does not reflect sound waves but by replacing the medium with water containing nylon microspheres (Orgasol 3501, EXD, Atochem, Newbury, Berks., U.K.) (Oates 1991) it was possible to calculate the flow rate with the 'Op Dop'. However, this method was only really practical for calibrating the system as the use of microspheres in the system might interfere with the process of intimal hyperplasia in the vein culture. The second, simpler method was to disconnect the tubing and empty the fluid pumped by the system in one minute into a measuring cylinder. This method was applicable at the end of each experiment but obviously could not be used to monitor flow throughout the experiment. However, as the pump rate and tubing diameter were kept constant throughout, there is no reason to expect any wide variations in flow. These 2 methods gave very similar results. It was found that using

Figure 7b.6 The Watson-Marlow 503U peristaltic pump used to produce the pulsatile flow in the system

Figure 7b.7 'Op Dop' recording of medium passing through silicone rubber tubing. The average velocity measurement is 16cm/s, with a mean flow of 175ml/min.



silicone rubber pump tubing of 5.5mm internal diameter with the pump set at 30 RPM led to a flow rate of approximately 175ml/min (Figure 7b.7), a figure which is at the upper end of flow rates achieved in femoropopliteal bypass grafts (Chin *et al.*1988).

Variations in pressure, because of the effect on cyclic stretch, are also important in determining the effects of flow on cultured vein. Fluid pressure is determined by the flow rate and the resistance of the system. Thus, the speed of the pump and the tubing diameter are very important in determining the pressure in the system. In addition, the height of the reservoir above the vein is important. The higher the fluid level in the reservoir is above the height of the vein, the higher the pressure is. As the flow rate is fairly fixed, once the pump is set at a certain setting and as the resistance of the system is unlikely to alter during the experiment, pressure can be varied by altering the height of the fluid level. Pressure in the system could be measured using a pressure transducer. This was done with the assistance of Miss Abigail Thrush in the Department of Medical Physics, Leicester Royal Infirmary. It was technically easier to measure the pressure in the silicone rubber tubing just before it entered the vein chamber and just after it left the vein chamber, by inserting a needle into the silicone rubber tubing, rather than attempting to measure the pressure directly in the vein itself. In practice, there was never more than 5mmHg difference between the values obtained pre and post vein chamber, and a mean value was taken. An example of the pressure waveform produced by the pump is shown in Figure 7b.8. A mean pressure could be calculated and this could be plotted against the height of the level of fluid in the reservoir as shown in Figure 7b.9. This produced a straight line graph, and it was therefore easy to calculate the height of the reservoir required to produce a given pressure.

Figure 7b.8 An example of the pressure waveform produced by the flow rig. Whilst the waveform is dichotomous, there is a wider pulse pressure difference than there is between systolic and diastolic arterial blood pressure.

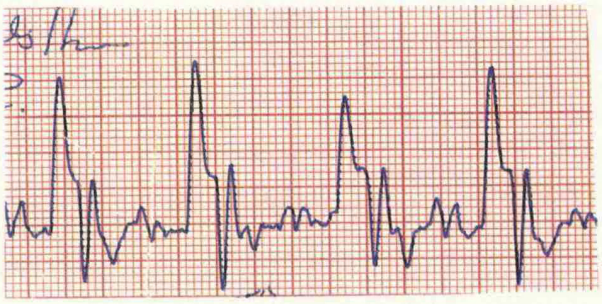
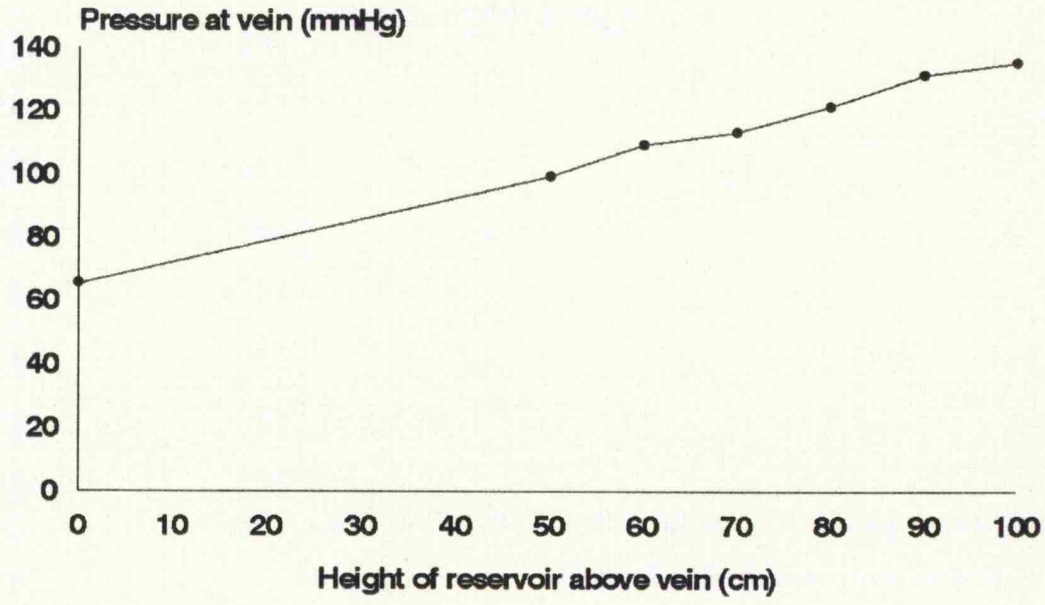


Figure 7b.9 A plot of mean pressure against height of the fluid column (height of the reservoir) above the vein.



The segment of vein was obtained as previously described in Chapter 6. The vein was harvested using a no-touch technique to minimise damage to the endothelium. A minimum length of 5-6cm was required to successfully complete the experiment. On arrival in the laboratory, the vein was carefully dissected free of excess fat and adventitia using fine surgical instruments. A 5mm segment of vein was assessed for endothelial integrity as previously described. Great care was taken not to damage any of the venous tributaries which were carefully ligated with 3/0 silk. This was essential to prevent any leakage of fluid outside the vein whilst it was being perfused. The vein was then carefully attached to the longer of the 2 metal cannulae in one of the vein chamber caps. The vein was secured with a 3/0 silk tie. Each cannula had a grooved internal end which facilitated the tying of a suture to hold the vein in place. The cannula was then pushed all the way through the cap and the cap was screwed on to the vein chamber. The other end of the vein could then be attached to the cannula in the other cap and was secured with a 3/0 silk tie before the cap was screwed on, taking care not to twist the vein. Once the vein was secured within the vein chamber, the chamber could be filled with medium and the vein could be added to the circuit. If there were any valves in the vein segment, then the direction of flow had to be arranged so that it did not cause the valves to close and oppose the flow. The external ends of the cannulae had Luer lock fittings which facilitated the attachment of the silicone rubber tubing.

At the end of the 14 day period of culture, the vein was removed, opened up longitudinally and divided into 3 segments using a no.23 scalpel blade. One piece was used for contractility studies to show that the vein was still viable. The other 2 pieces were pinned out in vein culture dishes in the same way that static vein cultures are set up. These 2 veins were then fixed overnight, one by adding 10% formalin to the vein culture dish and the other by adding 4% glutaraldehyde. The vein fixed in the formalin was sent off for histological processing and the other piece was processed for scanning electron microscopy. This involved washing with 0.2M cacodylate buffer and then dehydrating by adding to 50% through to complete alcohol in stages. The dehydrated vein was then placed in acetone, in a glass container, and sent for scanning electron microscopy.

7b.iii) DISCUSSION OF PROBLEMS WITH SYSTEM.

This model was not an unmitigated success. There were several problems concerning sterility, fluid leaks and gas flow. These problems, combined with the fact that it was not possible to obtain 2 equal lengths of vein from the same patient to compare using different flow conditions, rendered the model impractical for studying anything other than the effects of flow compared to no flow. Even then, because of logistical problems, it was not possible to complete more than 3 experiments successfully.

Infection is always a problem in tissue culture work. In this model, there were several items of equipment to combine together to set up the flow model. There were therefore several potential points at which infection could occur. Whilst the equipment was autoclaved and largely put together in a laminar flow hood using aseptic conditions, the vein chamber had to be placed in the incubator before it could be added to the circuit. Thus some of the tubing had to be connected in the open laboratory and some infections did occur. Such infections would usually manifest themselves after around 3 days of culture. The culture medium in the reservoir would become cloudy and there would be a sediment deposited on the bottom of the flask in excess of that normally seen. The commonest organisms were Gram negative bacilli, yeasts and other saprophytic organisms. However, the culture medium would often come back as stating 'No bacterial growth' despite the fact that it was cloudy with a 'musty' smell. The original antibiotic prophylaxis used in the culture medium was a combination of penicillin and streptomycin. This combination has poor efficacy against Gram negative bacilli and no effect on yeasts. This antibiotic combination was later supplemented with low dose gentamicin (See 7c)) with a subsequent reduction in the infection rate.

Leaks of culture medium were a constant problem. There were several points at which such leaks were likely to occur and, with practice, the frequency of such leaks diminished.

One of the earliest leaks that occurred was from the reservoir itself. As explained above, the system was not entirely closed as gas had to be allowed to escape. The gas was bubbled in very gently at a slow rate. However, in the first few experiments, the gas cylinder

would occasionally discharge itself completely overnight, leading to a huge rise in pressure within the reservoir with a resultant blowing off of the stopper in the reservoir and an explosion of medium over the bench. The reason for this sudden discharge of the gas was felt to be a rise in temperature of the gas within the cylinder. The gas cylinders were normally kept in a purpose-built storage room outside the main building. During the Winter months, the temperature was usually around 0-10°C. The cylinders were brought into the laboratory where the temperature was around 22°C. As the gas in the cylinder slowly warmed up, the pressure would rise and the gas flow rate would rise significantly. This would invariably happen at night time when there was no one in the laboratory to correct the situation. This problem was remedied by fitting a needle valve on the gas cylinder so that even if there was a significant rise in pressure, the gas flow rate remained constant.

Another potential site for leaks was the silicone rubber tubing within the pump chamber. The tubing is repeatedly compressed by the action of the pump rollers and does wear out with time. The tubing was moved every day so that the same parts of the tubing were not being worn away. If this was not done, the tubing could have worn away sufficiently for it to spring leaks (This occurred with one experiment only when a piece of tubing was re-autoclaved and the same length was inadvertently placed in the pump chamber again).

Each join in the system represents a potential site for a leak. Such leaks were minimised by having tight fits at each join. The most common site of leak within the system was at the vein chamber itself. The 2 stainless steel threaded caps had a rubber O-ring on their inner surface to help make the join as watertight as possible. Despite this O-ring, leaks still occurred quite frequently. The use of PTFE tape around the steel threads further improved the watertight seal, but there was still some potential for a leak. One of the most important changes made to prevent leakage was the addition of a silicone rubber tube leading from one of the offset portholes in the vein chamber to the return tube going back to the reservoir. This prevented the excessive build-up of pressure within the vein chamber which precipitated the leaks around the O-rings.

7 b.iv) RESULTS

Only 3 out of 6 veins successfully lasted 14 days in culture with no leaks or infections. All 3 of these veins maintained their normal architecture with normal endothelial coverage on scanning electron microscopy (Figures 7b.10 and 7b.11) and light microscopy (Figure 7b.12). In addition, the veins were able to contract in an organ bath in response to a depolarising solution of potassium chloride, demonstrating their viability.

Figure 7b.10 Scanning electron micrograph (503x) of luminal surface of vein after 14 days of flow conditions. The endothelial cells are morphologically intact.

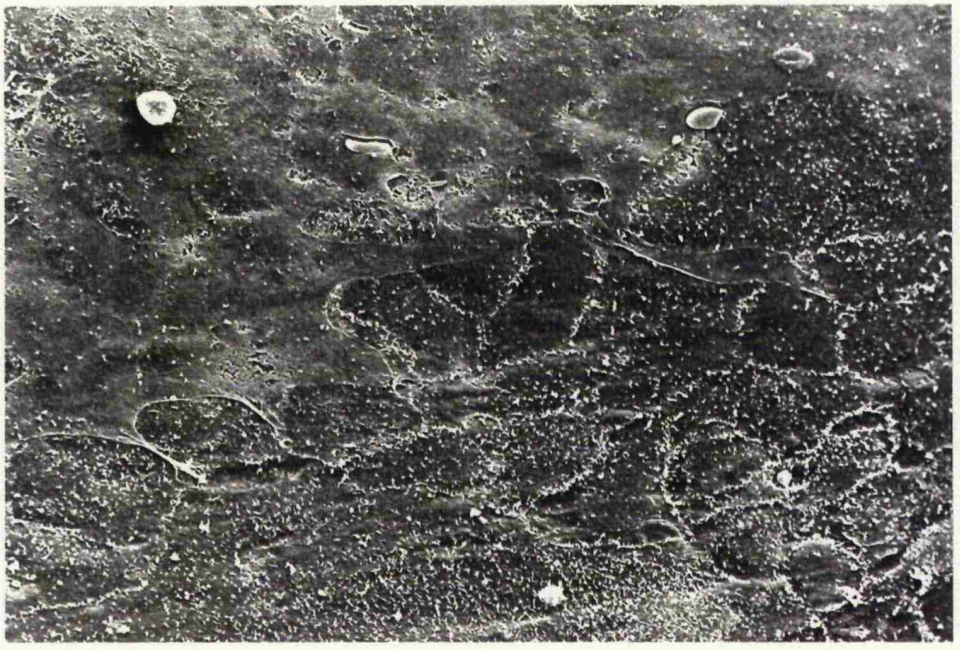


Figure 7b.11 Scanning electron micrograph (2530x) of vein shown above. The intact endothelial cells can be seen to be in close contact with one another.

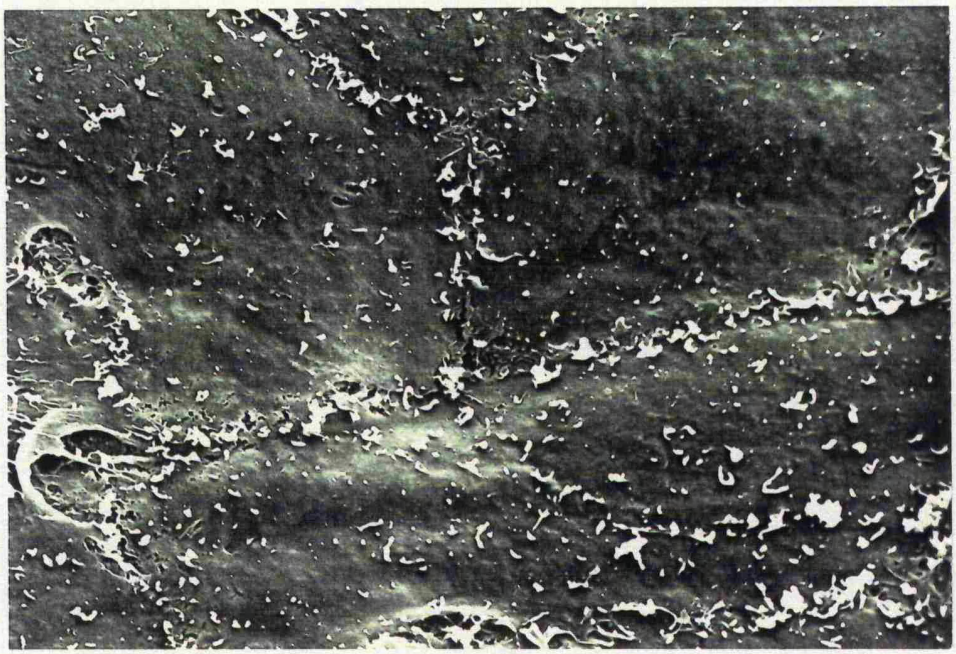
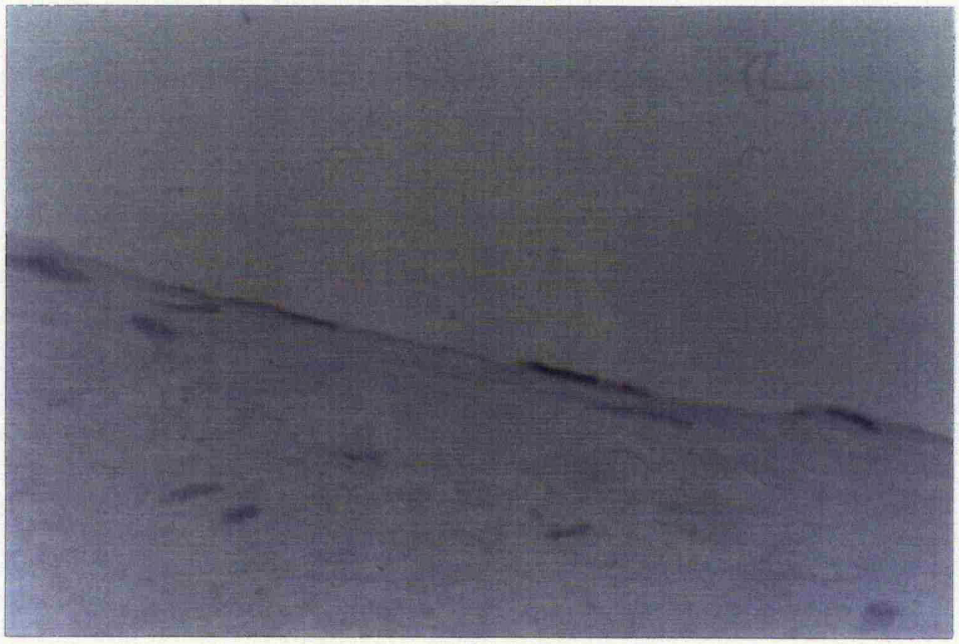


Figure 7b.12 Transverse section of cultured human saphenous vein after 14 days in flow x400 (CD31). The endothelial cells are stained brown where the monoclonal antibody to the CD31 antigen is present. A few endothelial cells are seen.



None of the cultured veins in the flow system developed any intimal hyperplasia despite the fact that they were in culture for 14 days and were still alive with an apparently intact endothelium at the end of the 14 day period. The control segments of vein in static culture did develop intimal hyperplasia. As there were only 3 successfully cultured flow model segments of vein, it is not possible to draw any valid statistical conclusions from the lack of intimal hyperplasia in these veins.

Figures 7b.13 to 7b.15 illustrate the histological findings of some of the veins from the flow model.

Figure 7b.13 Transverse section of cultured human saphenous vein after 14 days in flow x100 (H+E). The vein architecture does not appear to be altered.

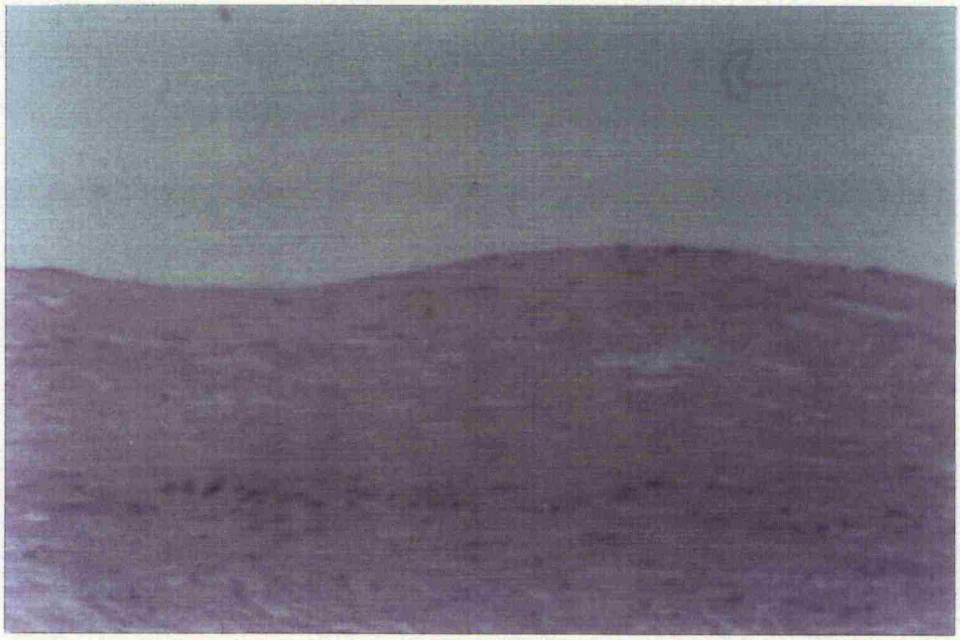


Figure 7b.14 Transverse section of cultured human saphenous vein after 14 days in flow x200 (smooth muscle and Miller's elastin). There is no intimal thickening visible on this section.

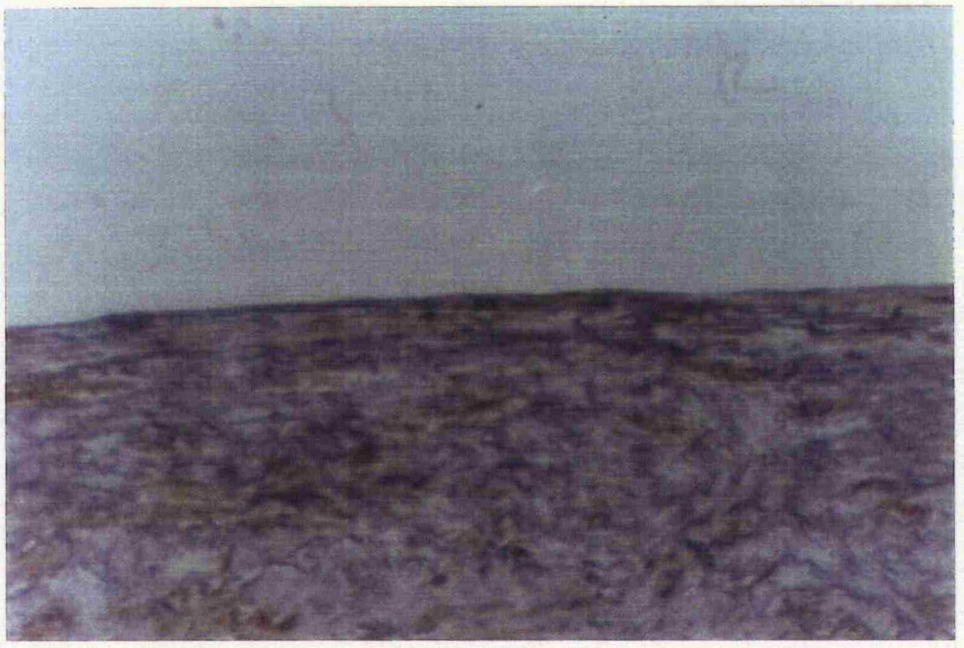
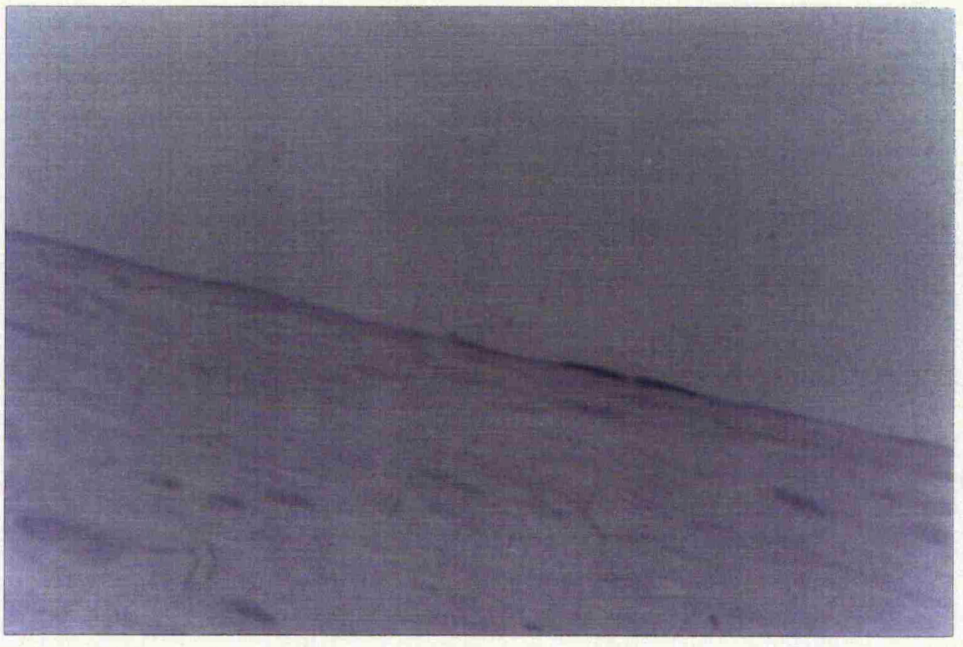


Figure 7b.15 Transverse section of cultured human saphenous vein after 14 days in flow x400 (BrdU). Two intimal cells are seen but neither one is taking up the BrdU.



7b.v) CONCLUSIONS

This flow model was not entirely successful. There were not enough successful experiments performed to draw valid statistical conclusions. Nevertheless, several veins were successfully cultured in arterial flow conditions for 14 days. Unlike the static vein cultures, these veins did not develop neointimal thickening over this time period. This was despite the fact that the endothelium remained morphologically intact, the normal vein architecture was maintained and the vein remained capable of contracting in the presence of potassium. Thus the effect of the flow rig appeared to be inhibitory on the development of intimal hyperplasia in cultured human saphenous vein.

An inhibition of intimal SMC proliferation is not unexpected in view of the wealth of evidence from *in vivo* animal studies (Faulkner *et al.* 1975, Rittgers *et al.* 1978, Morinaga *et al.* 1985, Morinaga *et al.* 1987, Dobrin *et al.* 1989, Kraiss *et al.* 1991, Hehrlein *et al.* 1991, Kohler *et al.* 1991, Geary *et al.* 1994a) and tissue culture studies (Yoshizumi *et al.* 1989, Sharefkin *et al.* 1991, Sterpetti *et al.* 1992c, Malek *et al.* 1993b, Kuchan *et al.* 1993) which suggest that high shear stress inhibits intimal hyperplasia whilst low shear stress promotes intimal hyperplasia. The effects of shear stress on intimal hyperplasia and on mitogen production by endothelial cells and SMCs have already been discussed in Chapter 2. The flow model also produces a cyclic stretching effect on the vein because of the pulsatile pressure. This might be expected to have a stimulatory effect on proliferation but this was not seen. The overall effect of the flow model appeared to be one of inhibition.

This model had several shortcomings, including the fact that a relatively long segment of vein was required. This meant that only one flow experiment could be performed with each segment of vein obtained. An ideal flow model to look at human saphenous vein would be one in which smaller segments of vein could be used so that parallel experiments using different flow regimes could be used on tissue obtained from the same individual.

7 c) THE EFFECT OF VARYING SHEAR STRESS ON INTIMAL HYPERPLASIA IN AN ORGAN CULTURE OF HUMAN SAPHENOUS VEIN.

7 c.i) INTRODUCTION

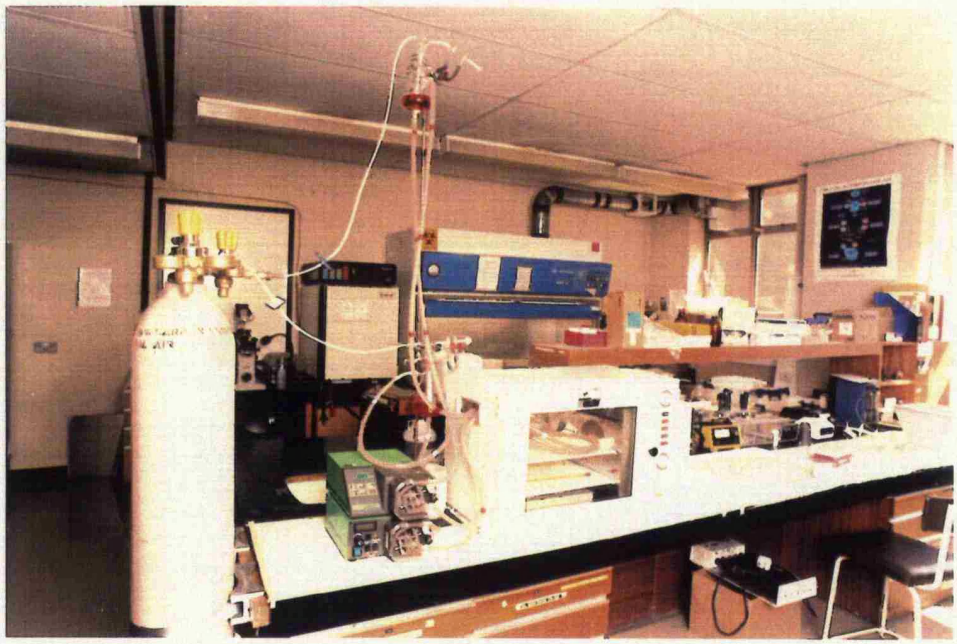
As explained in Chapter 2, flow is an important component in the development of intimal hyperplasia. High shear stress inhibits neointima formation in several animal and cell culture models whilst low shear stress potentiates SMC proliferation and the development of intimal hyperplasia. The flow model described in 7 b) had several shortcomings. In addition to the technical problems of fluid leakage and infection of the medium, the need for such a long segment of vein precluded setting up parallel segments of vein from the same patient under different flow conditions. The overriding consideration during the harvesting of human saphenous vein for research is the clinical need of the patient, not the requirement of vein for the research. Our ethical committee had given permission for the use of redundant segments of vein left over after arterial bypass surgery. This did not justify an extra incision for the harvesting of tissue. Thus to study human saphenous vein, a model was required that produced fewer technical problems and was able to subject relatively small segments of vein to different flow conditions. One way of doing this would be to use small segments of opened out vein placed in a system where they are exposed to flow conditions. This method is more comparable to the static culture.

7 c.ii) METHODS

A method was developed to allow 2 flow systems to run in parallel to compare the effects of different shear rates on the development of intimal hyperplasia in the human saphenous vein organ culture model. The flow system was similar to that described in 7 b). The reservoir, peristaltic pump, incubator, gas inflow and outflow and tissue culture medium were the same. However, the vein chamber was replaced by a 90cm length of silicone rubber tubing of 1cm internal diameter. The model is shown in Figure 7c.1. The vein segments were pinned out inside this length of tubing. A longitudinal slit approximately 10cm in length was made in the tubing to allow the veins to be placed inside. The veins were placed on pieces of shaped

500μm polyester mesh and pinned to the silicone tubing using A1 minuten pins (Watkins and Doncaster, Cranbrook, Kent). Once the vein was securely in place, the pins were cut short so that they did not protrude excessively into the lumen of the tubing. The veins were immersed in standard vein culture medium until the slit was resealed. Resealing was effected by using several layers of a proprietary silicone bathroom sealant whilst opposing the edges of the slit with a surgical tie around the tubing (3/0 nylon). Each layer of sealant was applied after the previous one had dried (~20 minutes). The medium covering the veins was replaced frequently whilst the sealant was drying. This procedure was performed within the laminar flow hood to maintain sterility.

Figure 7c.1 Flow model used to culture parallel segments of vein in arterial and venous flow conditions.

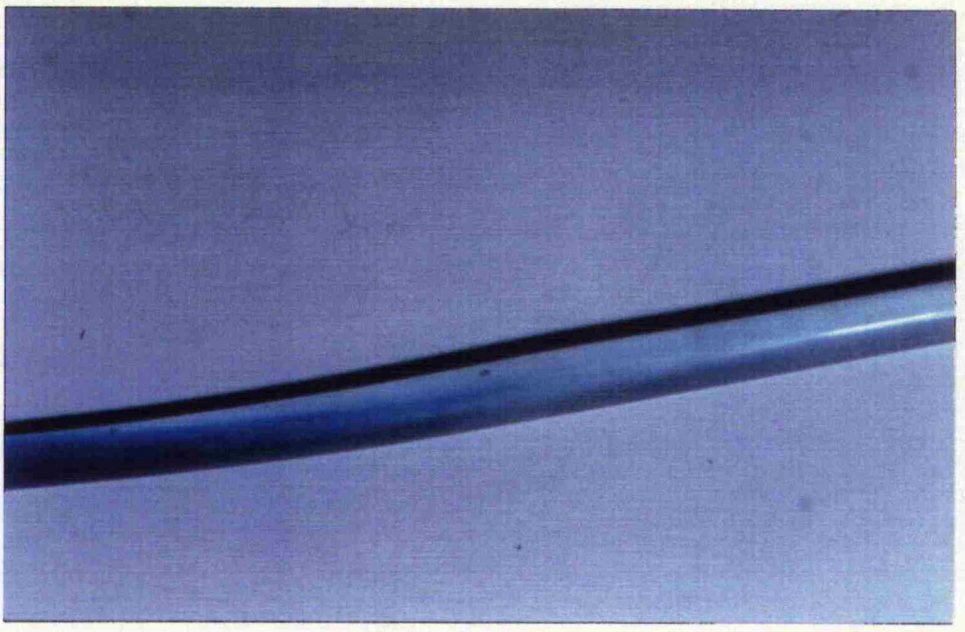


The reservoirs, filled with medium, were connected to the remainder of the circuit in the laminar flow hood under aseptic conditions. The lengths of silicone tubing containing the vein segments had to be connected to the circuit outside the laminar flow hood, where there was a potential for infection. The medium was then pumped around the system. This was

initially performed very gently to remove any air bubbles from the tubing and to make sure that there were no leaks in the system. There were occasional problems with leaks from around the slit in the silicone tubing but with practice these became progressively rare. The pumps were not set at their maximal rate until the following morning.

Whilst ideal laminar flow is almost impossible to obtain, a close approximation can be achieved providing the length of tubing proximal to the vein is at least 10x the diameter of the tubing. This was the case in this set of flow experiments. As a rough guide to whether the system approximated laminar flow, a flow rig was set up containing only water. Two mls of trypan blue were then injected rapidly via a hypodermic needle, some 15-20cms upstream from the site where the vein would be situated. The parabolic profile of the trypan blue shown in Figure 7c.2 is characteristic of laminar flow suggesting that the system was a good approximation.

Figure 7c.2 Silicone tubing containing water after injection of a bolus of trypan blue dye. The parabolic profile of the trypan blue is characteristic of laminar flow.



The vein was obtained from the usual source (Chapter 6) and was brought to the laboratory as quickly as possible. The vein was dissected free of excess fat and adventitia, a sample was tested for endothelial integrity and the remainder was divided into 6 segments for use in each experiment. Two segments were placed in static culture, 2 in 'arterial' conditions and 2 in 'venous' conditions. The venous and arterial conditions referred to the pressure and the calculated shear stress rather than flow rates. The pump rates and the height of the reservoirs were adjusted to obtain the necessary pressures and shear stresses. Advice and help was again obtained from Miss Abigail Thrush in the Department of Medical Physics regarding the measurement of the various parameters. The viscosity of the vein culture medium was measured by the haematology laboratory in the Leicester Royal Infirmary. The 'arterial' system had a flow rate of 500ml/minute, a mean pressure of 85mmHg with a calculated shear stress of 9 dyne/cm^2. The 'venous' system had a flow rate of 70ml/min, a mean pressure of 15mmHg and a calculated shear stress of 1 dyne/cm^2. Thus the flow rates were higher than would be expected in a vein graft or in a normal vein, because of the larger diameter of the silicone tubing, but the pressure and shear stresses were of the same order of magnitude.

The veins were cultured for a total of 14 days in their respective culture conditions. At the end of this period, the portion of tubing containing the veins was isolated from the rest of the system and excised using a no. 23 scalpel blade. One vein from each flow system and one of the static veins were assessed for viability using contractility studies whilst the other flow veins were immersed in 10% formaldehyde whilst still pinned to the silicone tubing. The medium was removed from the remaining static cultured vein and was replaced with 10% formaldehyde. The veins were then prepared, sectioned and stained as previously described in Chapter 6.

Control of infection

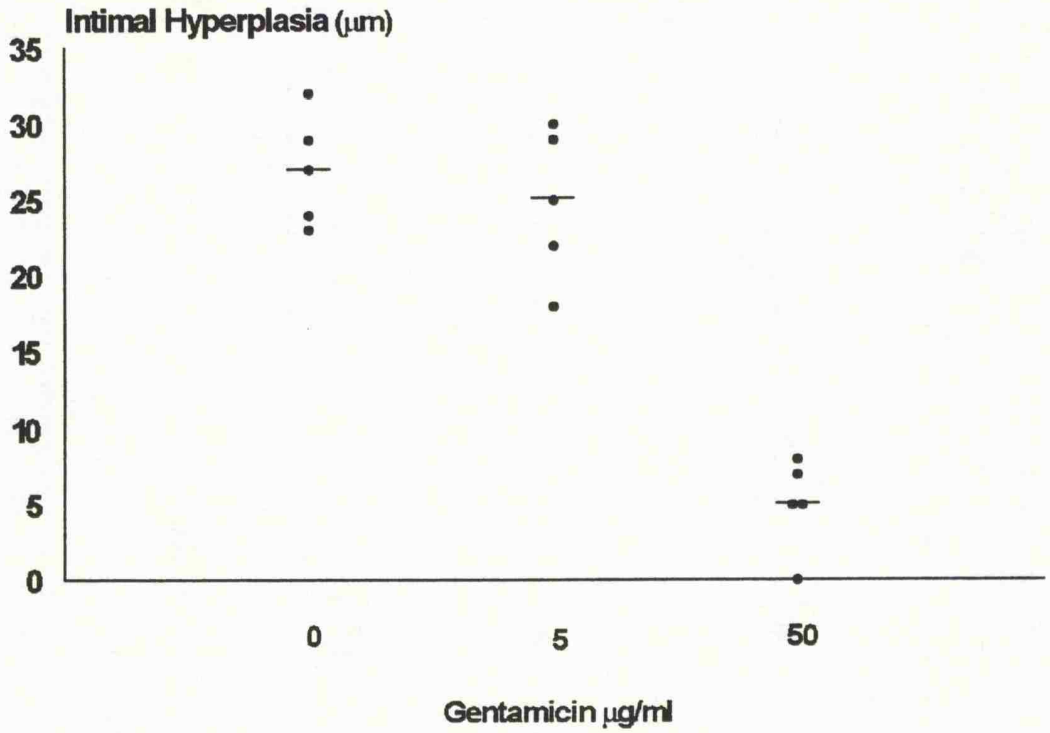
As with the original flow model described in 7 b), there were several infections early on in this flow model. These infections were invariably due to Gram negative bacteria which were resistant to the usual penicillin and streptomycin prophylaxis. Gentamicin is very effective against Gram negative infections but does have toxic effects on some cultured cell lines, notably corneal epithelium (Alfonso *et al.* 1990, Nelson *et al.* 1990, Medin 1993), keratinocytes

(Cooper *et al.* 1990) and foetal rat intestine cells (Shimizu *et al.* 1991). As I could not find any documented evidence of it having a toxic effect on vascular smooth muscle cells (nor could the manufacturers, Roussel), a toxicity study was performed.

Segments of saphenous vein were obtained from 5 different patients, each segment being long enough to obtain 3 samples for vein culture. The vein segments were prepared as previously described and were assessed for endothelial coverage. The veins were then divided into 3 pieces which were cultured either in standard vein culture medium, standard medium with gentamicin sulphate (Roussel Ltd., Dublin, Ireland) at 5μg/ml or standard medium with gentamicin sulphate at 50μg/ml. The veins were cultured for 14 days and were then fixed as previously described and stained for smooth muscle actin and Miller's elastin (Chapter 6). The neointimal thicknesses developed by the veins in culture are demonstrated in Figure 7c.3.

There was no significant difference between the control veins and the veins with 5μg/ml of gentamicin but there was a significant difference between the control veins and those veins cultured with gentamicin at 50μg/ml ($p=0.01$, Wilcoxon matched pairs signed Ranks test). The higher concentration of gentamicin had an inhibitory effect on the intimal proliferation. As the lower dose of gentamicin had no significant effect on the intimal hyperplasia, it was used routinely in the flow experiments.

Figure 7c.3 Scatter plot showing the effect of gentamicin on the neointimal thicknesses developed by human saphenous veins in culture.



7c.iii) RESULTS

Five experiments were successfully performed. In all of these experiments, the veins in flow and in the static culture remained viable after 14 days, as determined by the ability to contract when exposed to a depolarising solution of potassium chloride. Those veins in the static culture developed a median intimal thickness of 20μm, a figure similar to that obtained from the intact cultured veins in Chapter 6. The veins exposed to venous shear and pressure developed a median neointima of 8μm and those under arterial flow conditions developed a median neointima of 0 μm. These results are demonstrated in the scatterplot in Figure 7c.4. Representative slides are shown in Figure 7c.5.

The intimal hyperplasia developed by the vein under arterial conditions was significantly less than that developed by the vein in the static culture ($z = -2.02$, $p=0.04$, Wilcoxon signed ranks Log Rank test).

Figure 7c.4 Scatterplot showing intimal thickness developed by the veins in static culture and culture under arterial and venous flow conditions.

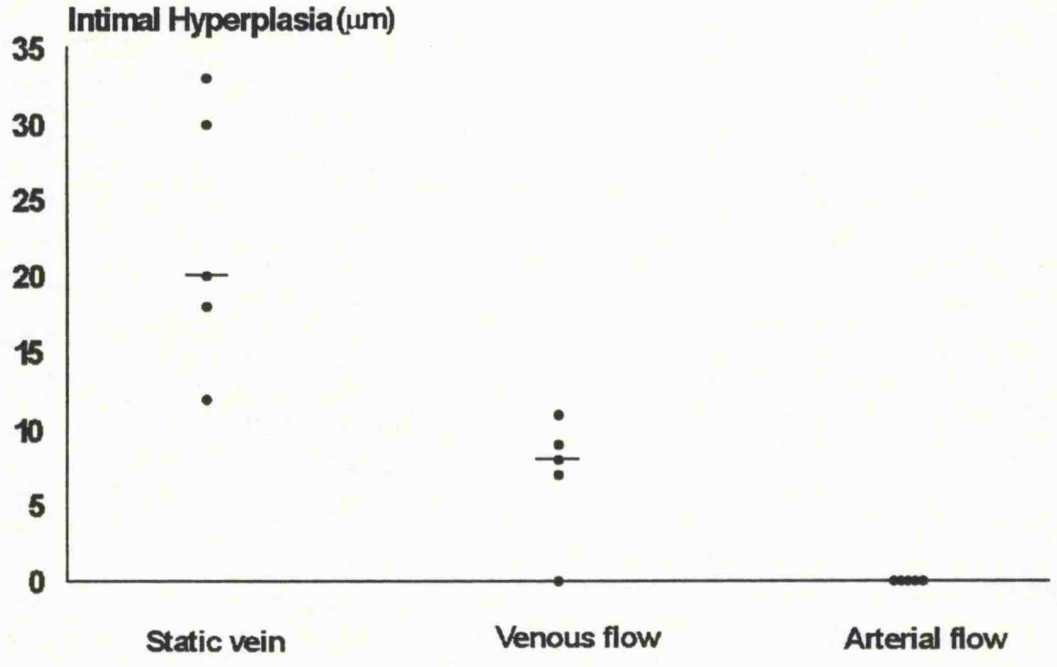


Figure 7c.5 Saphenous vein after 14 days in culture in static conditions x200 (smooth muscle and Miller's elastin).

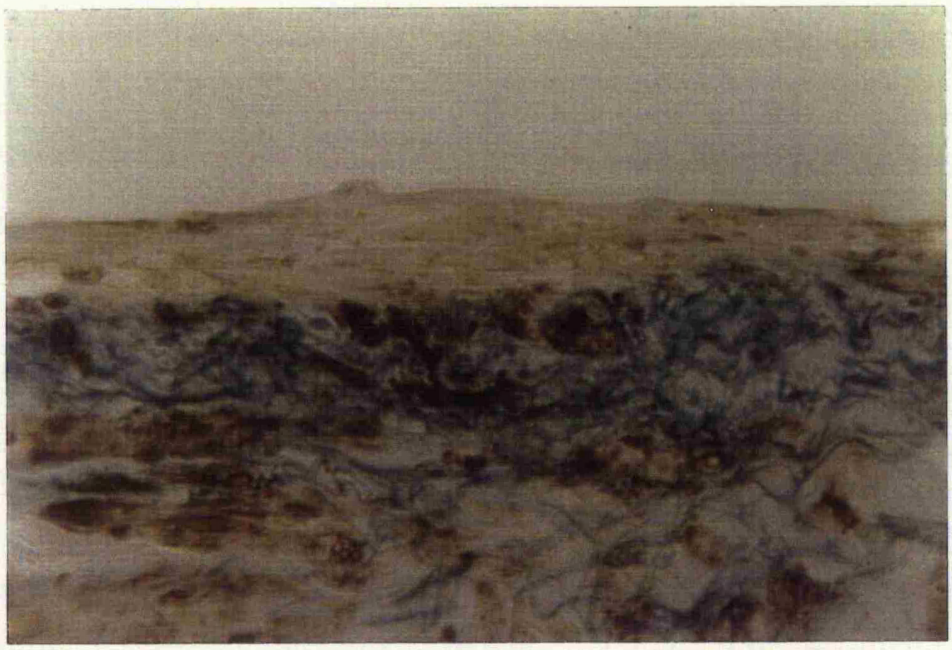


Figure 7c.6 Saphenous vein after 14 days in culture in venous conditions x200 (smooth muscle and Miller's elastin).

Figure 7c.7 Saphenous vein after 14 days in culture in arterial conditions x200 (smooth muscle and Miller's elastin).

7 c.iv) CONCLUSIONS

This experiment has confirmed the earlier findings from 7 b) that arterial flow conditions have an inhibitory effect on the intimal hyperplasia developed in cultured human saphenous vein. In this experiment, shear stress appears to have a graded effect. 'Arterial' shear abolished intimal hyperplasia entirely whilst 'venous' shear reduced intimal hyperplasia to a lesser extent. This effect correlates well with some of the animal and cell culture work reviewed in Chapter 2. Increasing shear stress has an inhibitory effect on SMC proliferation whilst low shear stress and, in the case of the static culture model, no shear stress promotes intimal SMC replication.

This effect has important implications for saphenous vein bypass grafts. The development of vein graft stenoses, due to intimal hyperplasia, in these grafts represents the commonest cause of graft failure between 1 month and 1 year after bypass surgery. Thus a better understanding of why these grafts develop stenoses may help us to reduce their incidence. Vein graft stenoses are discrete areas of intimal hyperplasia. These may develop at

areas of flow disturbance, where there is boundary layer separation with areas of reduced shear close to the vessel wall. It is thought that the intimal hyperplasia which leads to atheroma occurs in this way (Fox *et al.* 1966). It is in these areas of low shear stress that atherosclerotic plaques in carotid (LoGerfo *et al.* 1981b) and coronary arteries (Asakura *et al.* 1990) occur. Models of both end to side (LoGerfo *et al.* 1979, Crawshaw *et al.* 1980) and end to end (Kim *et al.* 1993) vascular anastomoses also reveal boundary layer separation with the areas of lowest shear stress occurring at the sites most prone to anastomotic intimal hyperplasia. A prospective study of early vein graft surveillance showed that areas of early flow disturbance were linked to the later development of vein graft stenoses (Mills *et al.* 1995). Thus the results of the flow model are in keeping with the published evidence that intimal hyperplasia is more common in areas of low shear stress whilst high shear stress has an inhibitory effect on SMC proliferation.

Chapter 8 - Summary, Conclusions and Future work

SUMMARY

Autologous saphenous vein remains the conduit of choice for peripheral arterial bypass surgery. Our own long-term results of peripheral bypass surgery were discussed in Chapter 4. In this group of patients there was a 7% perioperative mortality and a 20% perioperative graft occlusion rate. These early failures are usually due to technical errors whilst later graft failures usually follow the development of graft stenoses due to intimal hyperplasia. In this series, 34% of the patients developed at least one graft stenosis detected by the surveillance clinic during follow up. Although most of these stenoses were amenable to treatment with PTA, there was a subsequent high restenosis rate, with some 42% of the stenoses recurring at a median interval of 8.5 months. The principal pathology underlying restenosis is intimal hyperplasia. The development of stenoses at the anastomoses of synthetic grafts is also a result of intimal hyperplasia (Clowes *et al.* 1986). These stenoses are an important cause of failure in PTFE grafts but, as described in Chapter 4, a surveillance programme to look for these stenoses does not significantly improve graft patency.

Intimal hyperplasia is characterised by migration and proliferation of vascular SMCs. There are various growth factors known to have an effect on this proliferation in animal models. The effect of several of these growth factors on cultured human vascular SMCs was investigated in Chapter 5. These SMCs were derived from saphenous vein, the most frequently used conduit in peripheral bypass surgery. Platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and to a lesser extent interleukin 1 alpha (IL-1α) had a significant mitogenic effect on the SMCs. The proliferation achieved by PDGF was by far the most significant, approaching that of the 15% FCS medium itself.

The effect of PDGF on denuded human saphenous vein in organ culture was studied in Chapter 6 a). Despite the ability of PDGF to stimulate proliferation of isolated vascular SMCs, it had no effect on intimal SMC replication in denuded saphenous vein in organ culture. However in the coculture model in 6 b), where intact saphenous vein is able to stimulate

proliferation of intimal SMCs in the denuded vein (Allen *et al.* 1994), a blocking antibody to PDGF inhibits this proliferation. Thus PDGF appears to be necessary for the paracrine action seen in the coculture model, but it will not directly stimulate proliferation when added to a denuded vein. This implies another promoter of SMC replication, or a cofactor for PDGF, is produced by the intact vein in the coculture model but is not found in 30% FCS medium. The most likely candidate for this is endothelin which requires PDGF as a cofactor (Weissberg *et al.* 1990).

The conditioned medium from cultured human saphenous veins was found in 6 c) to stimulate the incorporation of tritiated thymidine into 3T3 fibroblast cells to a greater extent than culture medium containing 15% FCS. This effect was partially blocked by an antibody to PDGF. However, even after addition of the anti-PDGF, the conditioned medium still had a greater proliferative effect on the SMCs than 15% FCS, implying that another mitogen was present in the conditioned medium which was not present in the 15% FCS medium and was not inhibited by the anti-PDGF.

A segment of human saphenous vein was placed under arterial flow and pressure conditions whilst being perfused with tissue culture medium in the first part of Chapter 7. No intimal hyperplasia was seen after 14 days despite the fact that the vein still had a morphologically intact endothelium. Unfortunately, enough experiments were not performed with this model to draw any valid statistical conclusions although the arterial flow appeared to inhibit the intimal hyperplasia. The effect of shear stress on cultured human saphenous vein was confirmed in the second part of Chapter 7 where neointimal proliferation was inhibited to differing degrees by different shear stresses. Under 'arterial' conditions, intimal hyperplasia was completely abolished whilst under 'venous' conditions it was partially abolished. This suggests that the higher the shear stress, the greater the reduction in intimal hyperplasia. This has clinical relevance. Intimal hyperplasia is promoted by low shear stress. Graft stenoses may therefore be expected to occur where there are areas of low shear stress near areas of turbulent flow.

CONCLUSIONS

The cell and organ culture experiments suggest that PDGF is an important mitogen for human saphenous vein SMCs and that it probably plays a role in the proliferation seen in the organ culture model of human saphenous vein intimal hyperplasia. However, it is not the whole story and there are factors involved which may act synergistically with PDGF. Weissberg et al. demonstrated that endothelins were relatively poor mitogens when acting alone but were able to significantly stimulate vascular SMCs in the presence of PDGF (Weissberg *et al.* 1990). As endothelin is produced predominantly by the endothelium, it may well have been present in the coculture model and may have been responsible for part of the paracrine effect. The proliferative effect of endothelin would have been considerably reduced by the antibody to PDGF, as the paracrine effect was in Chapter 6. Further investigation of the role of endothelin in this model is currently underway in our Department. The development of endothelin antagonists may lead to agents for blocking the hyperplastic response of the SMCs in the vein culture model.

As discussed in Chapter 7, the organ culture model of human saphenous vein intimal hyperplasia is artificial in that the vein is bathed in culture medium in static conditions. These conditions may favour the development of intimal hyperplasia. As discussed in Chapter 2, high shear stress inhibits intimal hyperplasia in several animal models whilst low shear stress promotes it. The results from Chapter 7 show that increasing shear inhibits intimal hyperplasia in human saphenous vein. Vein graft stenoses are discrete areas of intimal hyperplasia. These may well occur at areas where there are flow disturbances with focal areas of low shear. This appears to be the case with anastomotic intimal hyperplasia (Bassiouny *et al.* 1992), although it has not been shown in graft stenoses. Indeed, a paper by Moody et al. suggests that there is no obvious reason why vein graft stenoses develop at particular sites (Moody *et al.* 1992a). They found no relation to sites of graft injury or apparent flow disturbance.

FUTURE WORK

Whilst PDGF is an important factor in the vein culture model of intimal hyperplasia, there is at least one and possibly several other factors involved in promoting neointima formation in the denuded vein in the coculture model. Endothelin is a very strong candidate as a promoter of intimal hyperplasia. Unpublished work from our laboratory suggests that endothelin is capable of producing intimal hyperplasia in cultured denuded saphenous vein. As endothelin requires PDGF as a cofactor, this fits in well with the results in Chapter 6. A blocking antibody to PDGF would inhibit the action of endothelin by removing its cofactor. Bovine PDGF is present in foetal calf serum and could act as a cofactor for endothelin in the vein culture model, allowing endothelin to promote intimal hyperplasia in a denuded vein. There are other experiments that can be done to look at the role of endothelin in this model. New endothelin antagonists are becoming available. Their effect on the development of intimal hyperplasia in cultured human saphenous vein can be studied. Similarly, blocking antibodies to endothelin could be studied in the coculture model to see if they are also able to inhibit neointima formation.

The advent of molecular biology has given rise to new techniques which may be used to study the vein culture model. In situ hybridisation techniques can detect whether mRNA for various growth factors is being expressed by cells in the cultured vein. These techniques are applied to prepared sections of the tissue after it has been cultured. Providing that appropriate oligonucleotides are used, they can be used to 'stain' for mRNA for growth factors such as PDGF, bFGF and endothelin. One can therefore see which cells in the cultured vein are expressing which growth factors and compare this to the vein prior to culture. Some early work has already begun in our laboratory, looking at the expression of PDGF in cultured human saphenous vein.

Molecular biology also provides another way of looking at the mechanisms involved in intimal hyperplasia in the vein culture model. Antisense oligonucleotides can be directed against mRNA for various growth factors and added to the vein culture medium. These oligonucleotides inhibit the translation of the mRNA message. Thus if one of the specific

antisense oligonucleotides inhibits the development of a neointima, it suggests that the message which is being inhibited is important in the process of intimal hyperplasia in the vein culture model. Antisense oligonucleotides to the proto-oncogenes c-myc (Bennett *et al.* 1994, Shi *et al.* 1994) and c-myb (Simons *et al.* 1992a) have been successful in inhibiting intimal hyperplasia in various animal models of intimal hyperplasia. As they are readily available, they could be applied to the human vein culture model, as could antisense oligonucleotides to various growth factors.

There are several avenues for further study of the flow model. Using the model described in 7 b), a range of different flow conditions can be applied. It is possible to compare the effects of laminar and non-laminar flow. The silicone tubing can be moulded to provide a number of different flow profiles. Thus it is possible to simulate the haemodynamic effects of a stenosis or a bifurcation. The response of human vascular tissue to various flow conditions can then be studied. A length of vein can be laid over an artificial stenosis and after culture any neointimal development can be assessed at different points over the stenosis. Such experiments could confirm whether intimal hyperplasia does occur in those parts of the vein where there is a lower calculated shear stress.

As with the static vein culture model, molecular biology techniques can be applied to the flow vein. In situ hybridisation techniques can be used to see which genes are being switched on or off in the flow vein in comparison to a static cultured vein or a freshly harvested specimen of vein. There may be suppressed expression of some growth-promoting genes in the flow vein. Analysis of such data may direct us towards genes to switch off in the static culture model or in *in vivo* experiments.

In addition to the laboratory work, there is now a clinical study underway looking at early flow disturbances following bypass surgery. Patients undergo completion scanning of vein grafts before discharge home so that early flow disturbances can be detected and followed up in the surveillance clinic. This will provide information as to whether these flow disturbances are associated with later stenosis development.

Appendix 1

SIMPSON'S RULE

$$A = dx*(y[0]+4*y[1]+y[2])/6$$

Where A = Area under curve

dx = Equally spaced intervals on the X axis

y = The Y values at each point

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